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Affinity purification and characterization of a key enzyme responsible for circadian rhythmic control of nyctinasty in *Lespedeza cuneata* L.

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Abstract—The synthesis of an affinity gel aimed at leaf-opening factor β-glucosidase (LOFG) and affinity purification of LOFG is presented. A gluconamidine-based β-glucosidase inhibitor was used as the ligand of the affinity gel. β-Glucosidase exhibiting an activity shift throughout the day was selectively purified from *Lespedeza cuneata* Don by the affinity gel. The resulting LOFG exhibited high substrate specificity toward the leaf-opening factor.

1. Introduction

A β-glucosidase is responsible for the storage and release of bioactive substances such as flavonoids, steroid saponins, and plant hormones in plants.[1,2] Glucosylation is thus important in controlling the concentrations of numerous bioactive substances in vivo. These compounds are converted to their corresponding β-glucosides and stored in the vacuole where their internal concentration is strongly affected by the activity of β-glucosidase.

Recently, the glucosylation–deglucosylation cycle has been confirmed as being involved in the control of the circadian rhythms in plant leaf-movement.[3,4] Leguminous plants close their leaves at night and open them in the morning according to the circadian rhythm. This rhythmic movement of the leaves is referred to as nyctinasty, which is controlled by a pair of leaf-movement factors, leaf-opening factor (LOF) and leaf-closing factor (LCF) (Fig. 1).[3,4] In *Lespedeza cuneata*, potassium lespedezate (**1**)[5] and potassium D-idarate (**2**)[6] have been identified as the LOF and LCF, respectively. Diurnal variations in the relative endogenous concentrations of LOF and LCF produce the rhythm in nyctinasty: the level of **1** in the plant body is controlled by the metabolic enzyme, leaf-opening factor β-glucosidase (LOFG), whereas that of **2** remains nearly constant during the day (Fig. 1).[6] This implies that LOFG plays a key role in the diurnal control of nyctinasty. In this paper, we describe affinity purification and some biochemical properties of LOFG.

2. Results and discussion

2.1. Syntheses of affinity gels with glucosidase inhibitor ligands

Affinity chromatography[7,8] has been used extensively for the purification of enzymes. In some instances, inhibitors of the corresponding enzymes have been used effectively as affinity ligands. For example, the amidine-type β-glucosidase inhibitor[9] β-glycosidase, which is also found in nature,[10–12] has been developed by Ganem,[13–15] Tatsuta,[16] Wong,[17] Heck et al.,[18] and Hiratake co-workers[19,20] In particular, Ganem's glyconamidine (**3**) and Hiratake's glycosylamidine (**4**) are both strong inhibitors of β-glucosidase at micromolar levels. Glycosidases are classified into 90 families[21] and each family contains a number of glycosidases with different substrate specificities; several of the β-glucosidases exhibit strong substrate specificity due to the structure of the aglycon moiety. Thus, for affinity purification of substrate-specific glycosidase, a glycosidase inhibitor with a specific aglycon structure is required as a ligand. We designed

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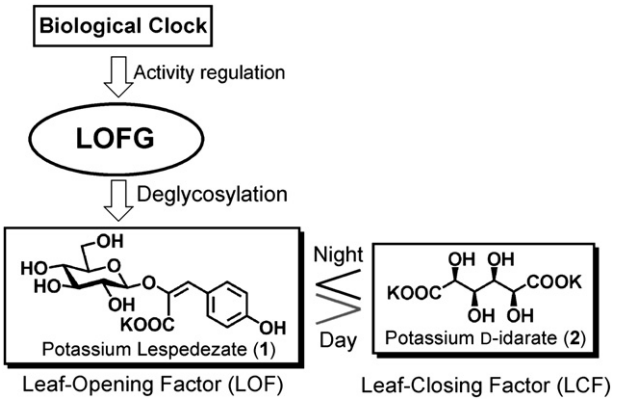
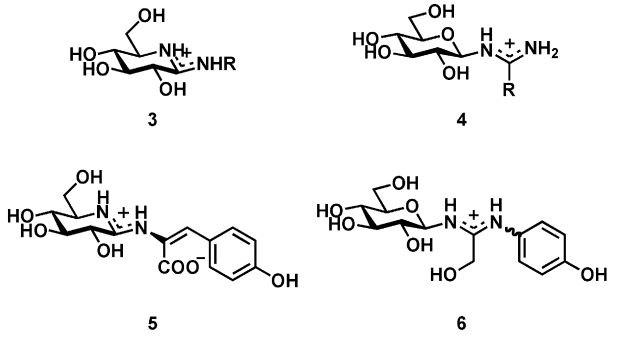


Figure 1. Leaf-opening factor β-glucosidase (LOFG) controlling the balance between the concentration of two leaf-movement factors (**1** and **2**) in the plant body.

β-glucosidase inhibitors **5** and **6** based on the structure of leaf-opening factor **1**. These two amidines are expected to function well as ligands for affinity purification of LOFG.

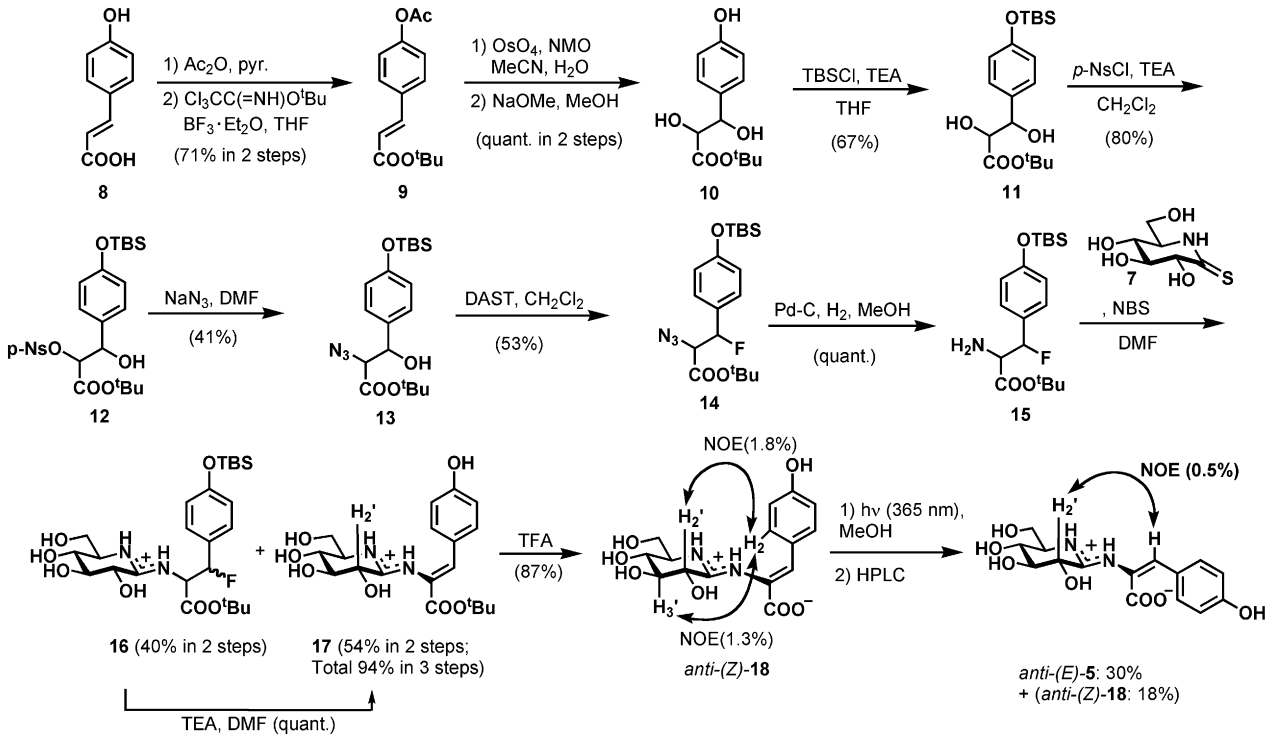


Synthesis of N^1-alkyl gluconamidine **5** was carried out as shown in Scheme 1. Syntheses of N^1-alkyl glyconamidine were reported by Ganem[15] and Vasella.[22–24] However, these known methods produced a mixture of gluconamidine and mannoamidine. The mannoamidine, an epimer of gluconamidine, was produced under basic coupling conditions through epimerization of the acidic 2′-H in the resulting gluconamidine. We developed a concise method for the synthesis of N^1-alkyl glyconamidine[25] using a non-protected thionolactam (**7**) prepared according to Vasella's method (Scheme 1).[22–24] *p*-Hydroxycinnamic acid (**8**) was fully protected[26] and diol was introduced to the olefin moiety by osmium tetroxide to give **10**. After deprotection, the α- and β-hydroxyl groups in **10** were substituted with azide and fluoride, respectively, to give **14**. Reduction of **14** gave **15**, which was coupled with thionolactam (**7**) using *N*-bromosuccinimide (NBS) to give a mixture of **16** and **17**. Fluoride **16** can be converted to **17** with triethylamine (TEA). Coupling product **17** was obtained in total yields of 94% with formation of olefin and deprotection of TBS group. An excess amount of **15** (ca. 10 equiv) was essential for a good yield. When the methyl ester of the corresponding amine was used, the complex oligomer of the amine produced by self-condensation was obtained. The use of fluoride (**15**) was essential for obtaining the coupling product. When we used the corresponding bromide or chloride instead of **15**, the coupling reaction gave a complex mixture because of the instability of these substrates under basic conditions. Importantly, our method gave only gluconamidine without the by-production of mannoamidine. This was because the free hydroxyl group at the 2′-position of **17**, which exists as an alkoxide-form under the coupling condition, prevents the abstraction of the 2′-H from the resulting **17** and subsequent formation of undesirable mannoamidine. Deprotection of **17** with trifluoroacetic acid (TFA) resulted in good yields of **18**. The geometry of the olefin and the stereochemistry of the N^1-alkyl amidine moiety in **18** were confirmed to be *anti*-(*Z*) by NOE correlations between H_2 and $H_{2'}$, and H_2 and $H_{3'}$ (Scheme 1). Then, **18** was photoisomerized into a 1:1 mixture of (*Z*)-**18** and (*E*)-**5**, with the desired (*E*)-**5** was isolated by HPLC. The small NOE between $H_{2'}$ and olefinic H demonstrated that the geometry of olefin in **5** is (*E*) and the stereochemistry of the amidine moiety is *anti* (Scheme 1).

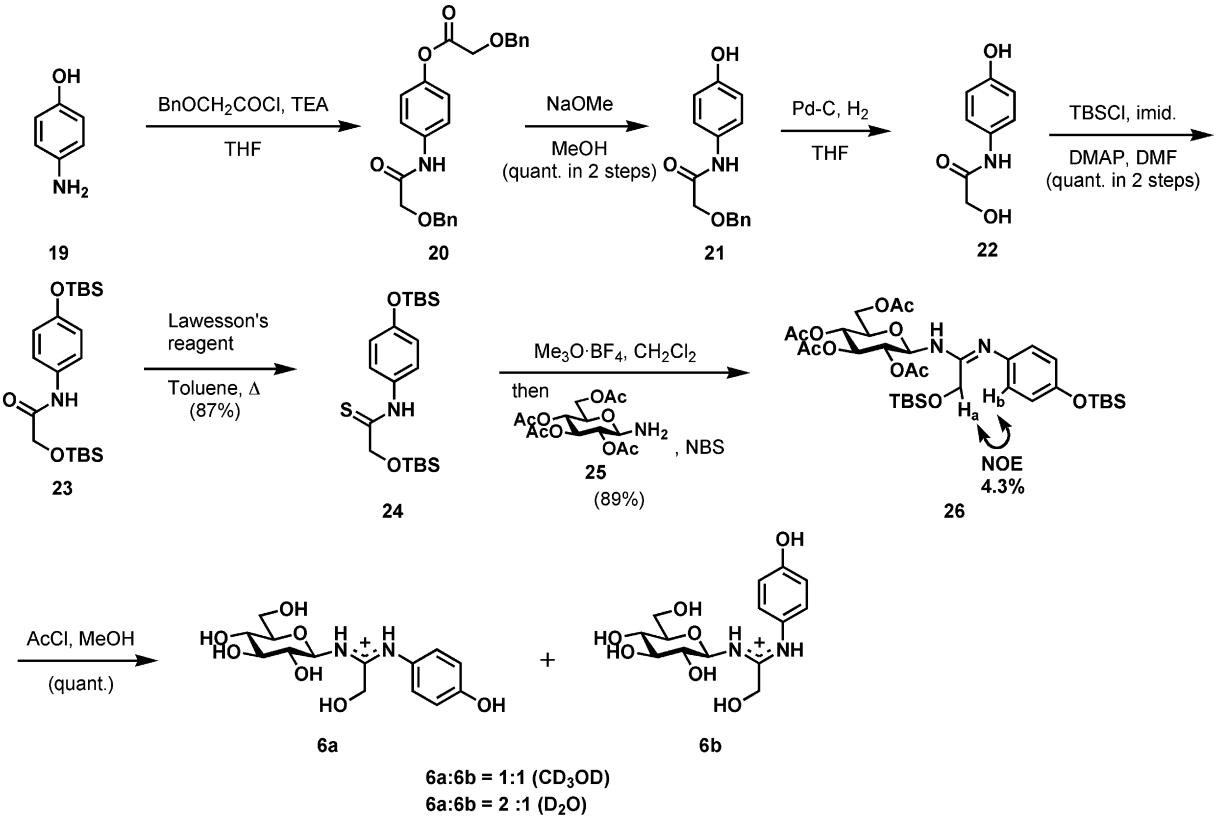
We also synthesized an N^1-alkyl glycosylamidine analog of **1** (**6a** and **6b**) according to a modification of Hiratake's method.[19] A thioamide aglycon (**24**) was synthesized from **19** as shown in Scheme 2. Then, formation of an N^1-alkyl glycosylamidine moiety was conducted using one-pot synthesis: methylation of **24** by Meerwein's salt and subsequent addition of protected β-glucosylamine (**25**) with NBS gave **26** as an *anti*-isomer. After deprotection, the N^1-alkyl glycosylamidine analog of **1** (**6a** and **6b**) was obtained as a mixture of *syn*- and *anti*-isomers, which isomerize easily and could not be isolated (Scheme 2). Our one-pot synthesis of the N^1-alkyl glycosylamidine using Meerwein's salt would be widely applicable to the syntheses of other glycosylamidine-type inhibitors.

We examined the inhibitory activities of synthetic amidine analogs (**5**, **6**, and **18**) against various glycosidases (Table 1). The K_i values were determined against several α-glucosidases and β-galactosidases using a Dixon plot.[27] Interestingly, a distinct difference was observed in the K_i values of (*E*)-**5** and (*Z*)-**18** against β-glucosidase from *Aspergillus niger*. The K_i value of (*Z*)-**18** was 5.8×10^{-5} M, whereas that of (*E*)-**5** was 1.6×10^{-6} M (Table 1). The inhibitory activity of **5** was 40-fold stronger than that of **18**. Additionally, the K_i value of **6** against β-glucosidase from *A. niger* was determined to be 1.3×10^{-6} M, which is as strong as (*E*)-**5**. Weak or no inhibition was observed against α-glucosidase for both **5** and **6**. Extremely high-specific inhibitory activity against β-glucosidase from *A. niger* was observed for **5** and **6**, whereas **18** inhibited β-glucosidase as strongly as α-glucosidase (Table 1); none of the amidines inhibited β-galactosidase. We developed micromolar β-glucosidase inhibitors (**5** and **6**) which are expected to be effective affinity ligands for the purification of LOFG. The inhibitory activity of each amidine against β-glucosidase varies with pH, with the optimum pH for **5** and **6** being 5.5 and 5.0, respectively (Table 2).

Stabilities of synthetic amidines are also important when applied as affinity ligands. We therefore examined the



Scheme 1. Synthesis of affinity gel with gluconamidine-type LOF **5**.



Scheme 2. Synthesis of affinity gel with glucosylamidine-type LOF **6**.

stabilities of **5** and **6** in aqueous solutions at a variety of pH values. Both **5** and **6** were extremely stable up to pH 6.5. However, **5** was unstable in an aqueous solution above pH 7, while **6** was stable over a wide range of pH from 4.5 to 9.0. These results suggest that compounds **5** and **6** are sufficiently effective as inhibitors and stable for practical application as useful affinity ligands under neutral or weakly acidic pH.

Table 1. Inhibitory activities of **5**, **6**, and **18** against glycosidases. K_i values were measured in 50 mM acetate buffer at optimal pH of each enzyme (pH 5.0 for β-glucosidases and β-galactosidase, pH 6.8 for α-glucosidase)

Enzyme	K_i (μM)		
	5	**6**	**18**
β-Glucosidase (from Almond)	255	Less than 90% inhibition at 1 mM	540
β-Glucosidase (from *Aspergillus niger*)	1.6	1.3	58
α-Glucosidase (from *Bacillus stearothermophilus*)	436	No inhibition at 1 mM	52
β-Galactosidase (from *Aspergillus oryzae*)	No inhibition at 1 mM	No inhibition at 1 mM	No inhibition at 1 mM

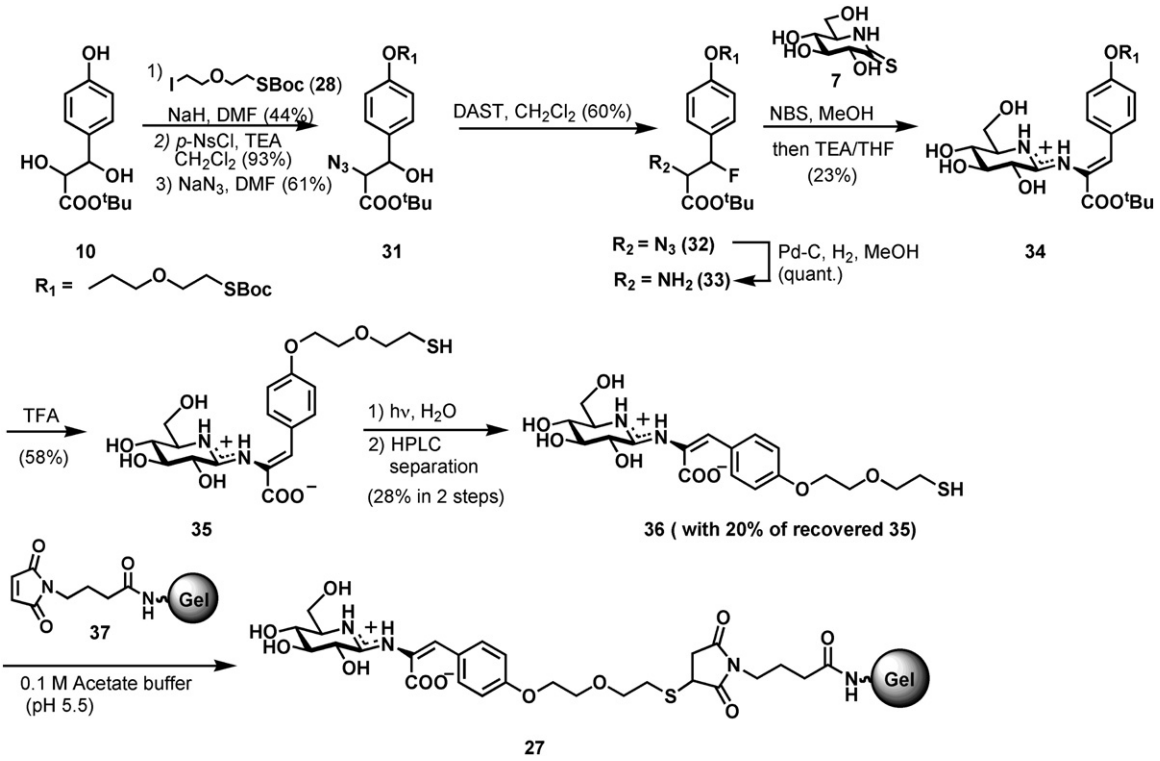
Next, we synthesized two affinity gels (**27** and **46**) with LOF-based inhibitors **5** and **6** as ligands (Schemes 3 and 4). Synthesis of gluconamidine-type affinity gel **27** is shown in Scheme 3. Affinity gel **27** has a linker moiety on the phenolic hydroxyl group which connects gluconamidine ligand **5** to the gel. Direct introduction of linker unit (**28**) to **5** gave a complex mixture because of the instability of the amidine moiety under coupling conditions. Therefore, the linker must be introduced before the formation of the amidine moiety. Compound **10** was coupled with linker **28** containing the protected thiol group. Then, the α- and β-hydroxyl groups were substituted with azide and fluoride, respectively, to give **32**. Reduction of the azide in **32** gave **33**, which was coupled with thionolactam (**7**) using NBS to give **34**. Successive deprotection and photoisomerization with UV light (312 nm) gave a 1:1 mixture of **35** and **36**, with the desired **36** obtained by HPLC purification. Stereochemistry of the olefin moiety of **36** was defined by comparison of spectral data with **5**. The synthetic ligand **36** was then coupled with gel **37** containing the maleimide moiety, which was prepared from the commercially available Toyopearl AF-Amino-650M, to give affinity gel **27**. Similarly, glucosylamidine-type affinity gel **46** was synthesized using a slight modification of the method used for **27** (Scheme 4) using Toyopearl AF-Carboxyl-650M.

Table 2. Inhibitory activity of **5** and **6** against β-glucosidase from *Aspergillus niger* under various pHs

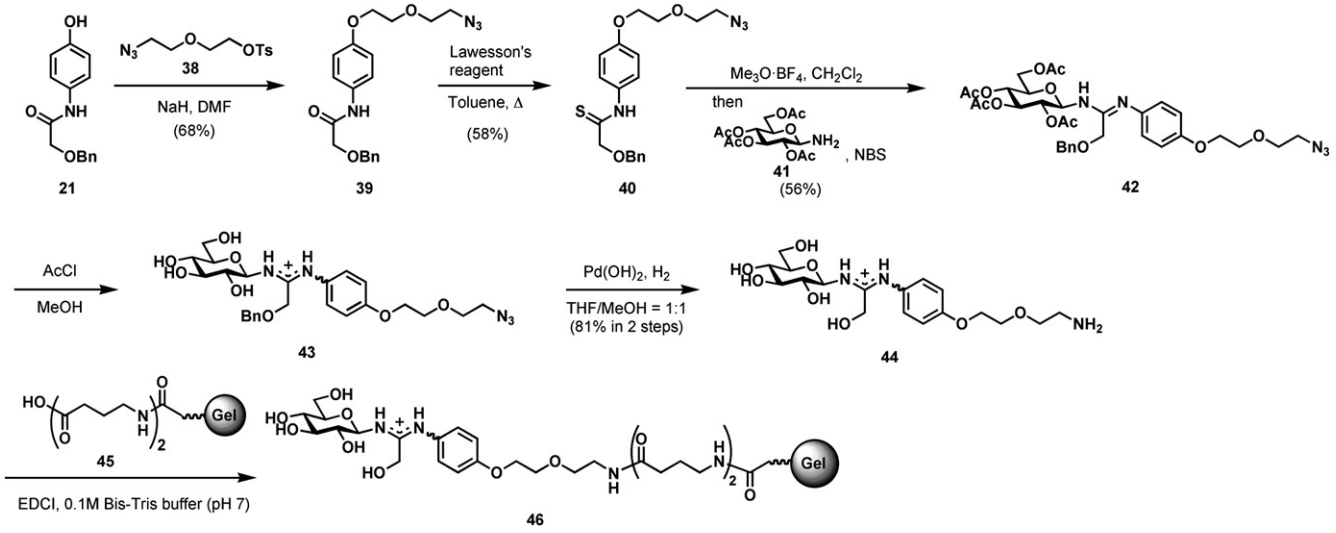
pH	K_i (μM)	
	5	**6**
4.5	1.64	3.50
5.0	1.60	1.30
5.5	1.51	1.33
6.0	12.9	4.12

2.2. Affinity purification of LOFG

We examined the affinity purification of LOFG from *L. cuneata* using affinity gels **27** and **46**. Preliminary purification of LOFG using **27** was partially communicated in Ref. 28.



Scheme 3. Synthesis of affinity gel with gluconamidine-ligand (**27**).



Scheme 4. Synthesis of affinity gel with glucosylamidine-ligand (**46**).

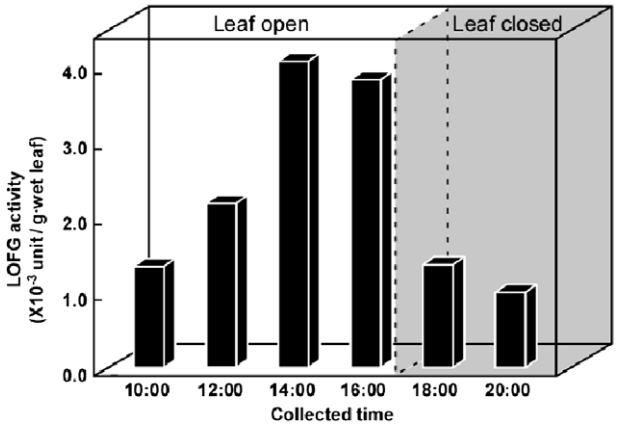
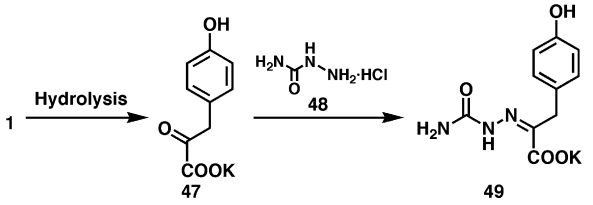


Figure 2. Diurnal fluctuation in β-glucosidase activity in the leaves of *Lespedeza cuneata* L.

Before initiating affinity purification, we established a reliable method for quantitative analysis of LOFG activity using **1** as a substrate (Scheme 5). α-Keto acid **47**, a hydrolytic product of **1**, was unstable and easily decomposed in aqueous solution, which meant that reproducible measurement of LOFG activity using **1** has so far been difficult. Therefore, we developed a novel method for the quantitative analysis of unstable α-keto acids. α-Keto acid **47** can be converted quantitatively into stable semicarbazone (**49**) (Scheme 5),[28,29]



Scheme 5. In situ formation of semicarbazone (**49**) from α-keto acid (**47**) for quantitative analysis of LOFG activity.

and HPLC analysis of **49** can be used to reliably measure β-glucosidase activity using **1**. All of the subsequent purifications are based on this analytical method.

Our previous study showed that LOFG activity in the plant body increased in the evening.[6] Therefore, it is important to determine when LOFG is activated in *L. cuneata* before attempting purification thereof. We prepared a series of acetone powders from the leaves of *L. cuneata* as a crude enzyme containing LOFG by collecting leaves every 2 h from 10:00 to 20:00 h. Each acetone powder was extracted with 0.1 M Bis–Tris buffer (pH 7.0), and LOFG activity of each extract was measured using **1** as a substrate. Analysis of **49**, which is formed by the in situ coupling of **47** and **48** in aqueous solution,[30] revealed a remarkable increase in LOFG activity between 14:00 and 16:00 h (Fig. 2). Thus, further purification was conducted using acetone powder prepared from the plants that were sampled between 14:00 and 16:00 h.

The acetone powder collected between 14:00 and 16:00 h was extracted with Bis–Tris buffer (pH 7.0), and the proteins were precipitated with acetone. The resulting precipitate was dissolved in Bis–Tris buffer (pH 7.0) and then partially purified by anion exchange chromatography. All fractions with β-glucosidase activity were collected and subsequently applied to an affinity column with gel **27** or **46**. After washing the gel with sodium acetate buffer (pH 5.0), sodium acetate buffer (pH 5.0) containing 10 mM **1** was applied to the gel to elute the adsorbed LOFG. No LOFG activity was eluted by D-glucose because of the strong affinity of the ligand with LOFG. Next, the fraction containing LOFG was applied to gel-filtration chromatography to remove **1**. The overall purification of LOFG is summarized in Scheme 6.

Purified LOFG using gel **27** showed a glucosidase activity of 4.8 U/mg protein against **1**; a 240-fold enhance-

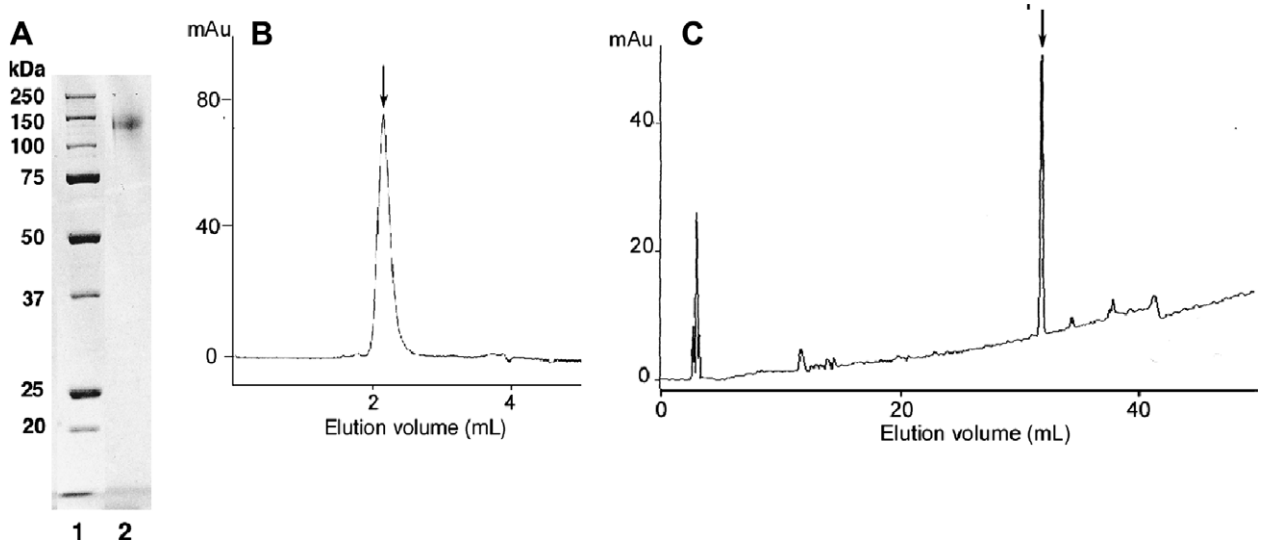


Figure 3. Purification of LOFG (A) SDS–PAGE analysis of LOFG [1: MW marker, 2: purified LOFG], (B) HPLC analysis of LOFG [column: TSK-Gel SuperSW3000; eluent: 50 mM phosphate, 150 mM sodium chloride buffer (pH 7.0)], (C) RP-HPLC analysis of LOFG [column: Sephasil protein C4; eluent: linear gradient from 2% CH_3CN–0.065% TFA to 65% CH_3CN–0.065% TFA in 50 min; flow rate: 1.0 mL/min; detection: 215 nm, temp: 40 °C].

Leaves of *L. Cuneata*
↓ Homogenize in cold acetone
Filtered and dried
Acetone powder
↓ Extraction
0.1 M Bis-tris buffer (pH 7.0)
2 mM DTT, 5 mM EDTA,1 M NaCl
Polyclar VT (8% w/v), ascorbic acid (0.125% w/v)
↓ Centrifugation (14000 g x 30 min)
Supernatant: Crude Enzyme
↓
Precipitation by Chilled Acetone (–20 °C, 60 v/v)
↓ Centrifugation (14000 g x 30 min)
Precipitate
↓ dissolved in 25 mM Bis-tris buffer (pH 7.0)
↓ Centrifugation (14000 g x 30 min)
Supernatant
↓
Anion-Exchange Column Chromatography
(HiPrep 16/10 Q XL)
↓
Affinity Column Chromatography by **27** or **46**
↓
Gel-filtration chromatography (Superdex 200)
↓
Purified LOFG

Scheme 6. Summary of purification of LOFG from the leaves of *Lespedeza cuneata* L. G. Don.

ment of activity compared with the first extract.[28] One enzyme unit of LOFG was defined as the quantity of enzyme required to hydrolyze one micro mole of **1** in 1 min under optimum pH conditions at 30 °C. LOFG of similar purity was also obtained using affinity gel **46**. SDS–PAGE analysis of the resulting LOFG gave a smear band at approximately 150 kDa (Fig. 3A), whereas this LOFG produced a single sharp peak in gel-filtration HPLC analysis using TSK-Gel SuperSW3000 (Fig. 3B) and RP HPLC analysis (Fig. 3C). The molecular weight of LOFG estimated from the elution volume in gel-filtration HPLC corresponded to that observed using SDS–PAGE.

The MW of normal β-glucosidase is approximately 60 kDa.[20,31–36] Since LOFG is considerably larger than normal β-glucosidase, we assumed that LOFG undergoes some type of post-translational modification. We examined the presence of the sugar chain on LOFG because many of the glycoproteins are known to exhibit a smear band on SDS–PAGE.[37–40] A modification of O'Shannessy's method[41] was employed for the detection of the sugar chain, and LOFG was subjected to SDS–PAGE with Western blotting on a polyvinylidene difluoride (PVDF) membrane. The membrane was first treated with 10 mM $NaIO_4$ and then with 0.1 mM biotinhydrazide to biotinylate the sugar chain of the glycoprotein. While chemiluminescence detection with streptavidin-horseradish peroxidase (HRP) conjugate clearly demonstrated the presence of a sugar chain on LOFG (Fig. 4), treatment of LOFG with *N*-glycopeptidase F,[42] which is commonly used for the deglycosylation of glycoproteins, did not produce any successful results (Fig. 5). We therefore examined the chemical dissociation of the sugar chain using trifluoromethane sulfonic acid (TfOH).[43,44] Purified LOFG was treated with TfOH and analyzed by SDS–PAGE. The smear

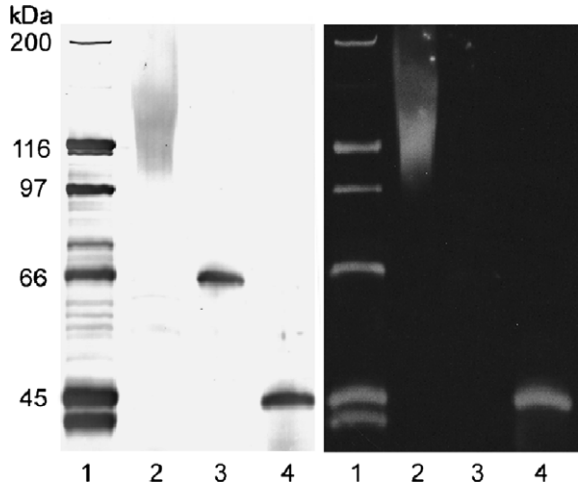


Figure 4. Detection of the sugar chain in LOFG. (Left) membrane stained by colloidal gold total protein stain (Bio-Rad Laboratories). (Right) biotinylated sugar chain detected by chemiluminescence (1, marker protein; 2, LOFG; 3, BSA as negative control; 4, ovalbumin as a positive control).

band produced by LOFG of approximately 150 kDa on SDS–PAGE shifted to a smaller, sharper band which at approximately 60 kDa. In addition, gel-filtration HPLC analysis of the TfOH-treated LOFG also produced a single, sharp peak at approximately 60 kDa (Fig. 5). Therefore, it was concluded that LOFG is a 60 kDa protein which was subjected to post-translational glycosylation by plural sugar chains whose total mass would be about 90 kDa.

We then sought to undertake the de novo sequence analysis of LOFG. In general, de novo sequence analysis by MALDI-TOF MS produces reliable results when a protein can be supplied in sufficient quantities and is detectable by Coomassie brilliant blue (CBB) staining. However, the amount of LOFG obtained using **27** was so limited that de novo sequence analysis was unable. In addition, affinity gel **27** gradually decomposed during use, and affinity to LOFG decreased abruptly due to repeated use. Although a large amount of gel **27** is required for large-scale purification of LOFG, the supply is limited because of the low total yield associated with the synthesis of **27**.

We compared the total synthetic yields of two affinity gels: ligand **36** was synthesized in 13 steps with a total yield of 0.4% from commercially available **8** (Schemes 1 and 3), while ligand **44** required only seven steps and had a total yield of 18% from commercially available **19** (Schemes 2 and 4). Therefore, for the large-scale purification of LOFG, we conducted large-scale synthesis of affinity gel **46** and used it instead of **27** because it was highly stable, sufficiently durable for repeated use, and could be supplied in sufficiently large quantities because of the high synthetic yield. We obtained 1.1×10^{-1} unit of purified LOFG using **46**, which was sufficient for further biochemical examination and de novo sequence analysis by MALDI-TOF MS.

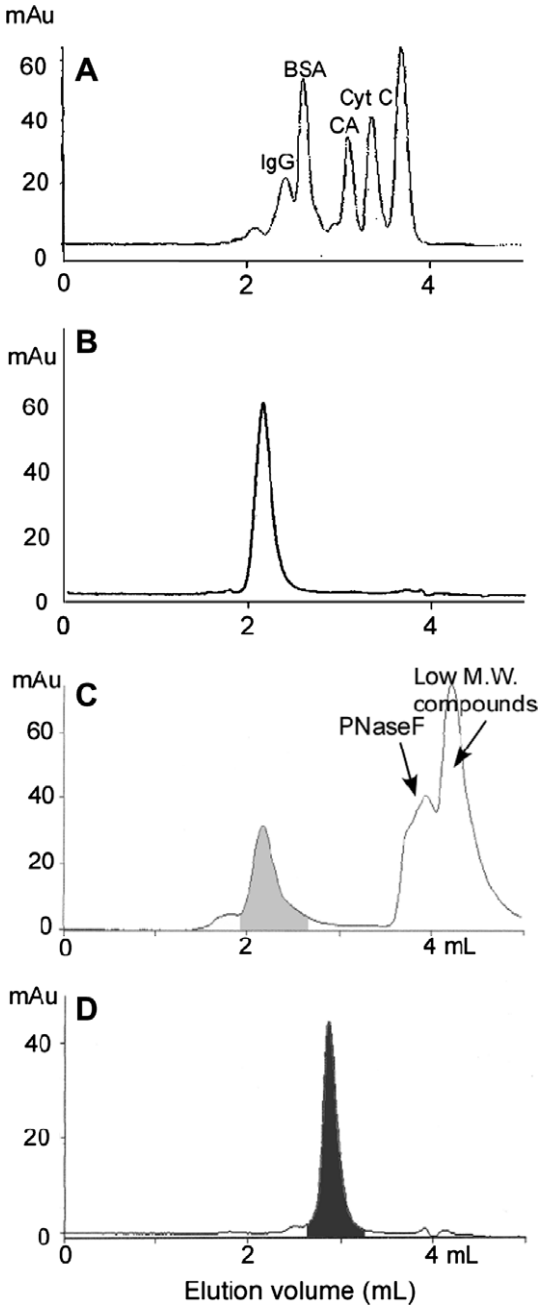
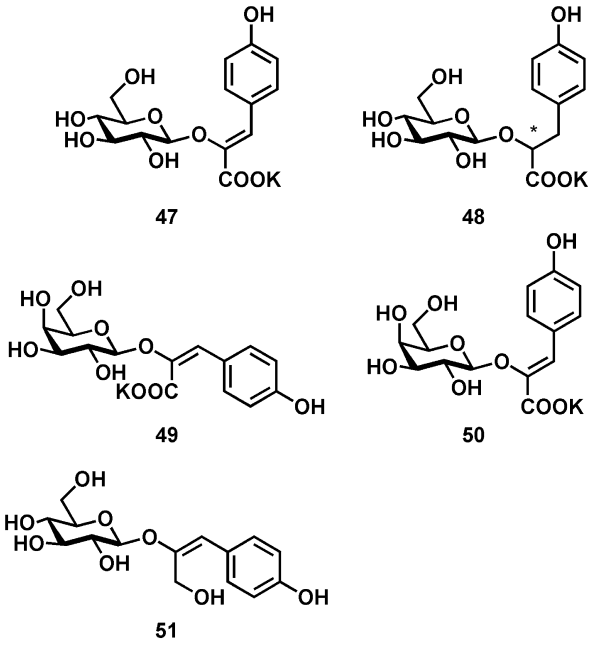


Figure 5. Gel-filtration analysis of LOFG by using TSK-gel Super SW3000 (A). Molecular weight standards [IgG: 150 kDa, BSA: 68 kDa, carbonic anhydrase (CA): 34 kDa, cytochrome *C* (CytC): 12 kDa], (B) intact LOFG, (C) LOFG treated with *N*-glycopeptidase F, (D) LOFG treated with TfOH.

Purified LOFG was hydrolyzed using trypsin in H_2O and $H_2{}^{18}O$, respectively, and the two hydrolyzates were analyzed by MS/MS. Digestion in $H_2{}^{18}O$ labels the C-terminal of the fragment peptide and enables differentiation between fragments based on the N- and C-terminals in the MS/MS experiment. Comparison of obtained data gave partial sequences from which the de novo sequence was elucidated (Table 3). The obtained partial sequences showed that the sequence of LOFG is highly similar to β-glucosidase of Family III.[21]

Table 3. Partial amino acid sequence of LOFG by de novo sequence analysis by MS/MS experiment

Entry	*m/z*	(N-Terminus) partial sequence from MS/MS (C-terminus)
1	1626	(511) (I or L) DEV (I or L) SA (I or L) (Q or K) K C
2	1827	(370) (RD)V (I or L) DCPV (Q or K) (I or L) SR
3	1727	(155) GFSY (I or L) SS PE VAF (I or L) R
4	1856	(574) Y (I or L) SS [PE or (I or L) (I or L)] VAF (I or L) R
5	1568	[(379)N or (396)P] VA (I or L) (83) (Q or K) GVYSR
6	1206	(Q or K) [D or (44)A] (72) AYYYVGR
7	1462	(271) V (Q or K) (I or L) G (196) V (74) (193) GR
8	1485	[(543)P or (526)N] E (Q or K) EHFR
9	1184	(214) (168) (I or L) (I or L) NPFAK or (214) (168) (I or L) (I or L) NDEAK
10	1018	(562) (I or L) VPK



We also examined the substrate specificity of the LOFG (Fig. 6 and Table 4). The K_m value of LOFG against **1** was determined to be 548 μM at pH 4.5, which is comparable to other substrate-specific β-glucosidases.[32–35,45] As shown in the comparison with potassium isolespedezate, LOFG recognized the olefin moiety of **1** (**47**)[5] and **48**[5] (Table 4). Potassium galactolespedezate (**49**)[46] and potassium galactoisolespedezate (**50**),[46] which have galactose instead of glucose, could not be hydrolyzed. On the other hand, the hydroxymethyl analog of **1** (**51**) was a good substrate for LOFG. These results showed that LOFG recognizes an aglycon moiety together with a glycon moiety, and that it showed high substrate specificity toward **1**.

Table 4. Relative activity of LOFG against various substrates

Entry	Substrate	Relative activity
1	Potassium lespedezate (**1**)	100
2	Potassium isolespedezate (**47**)	0
3	**48**	26
4	Potassium galactolespedezate (**49**)	0
5	Potassium galactoisolespedezate (**50**)	0
6	**51**	113

In conclusion, we have succeeded in the affinity purification of a candidate of LOFG, a key enzyme involved in

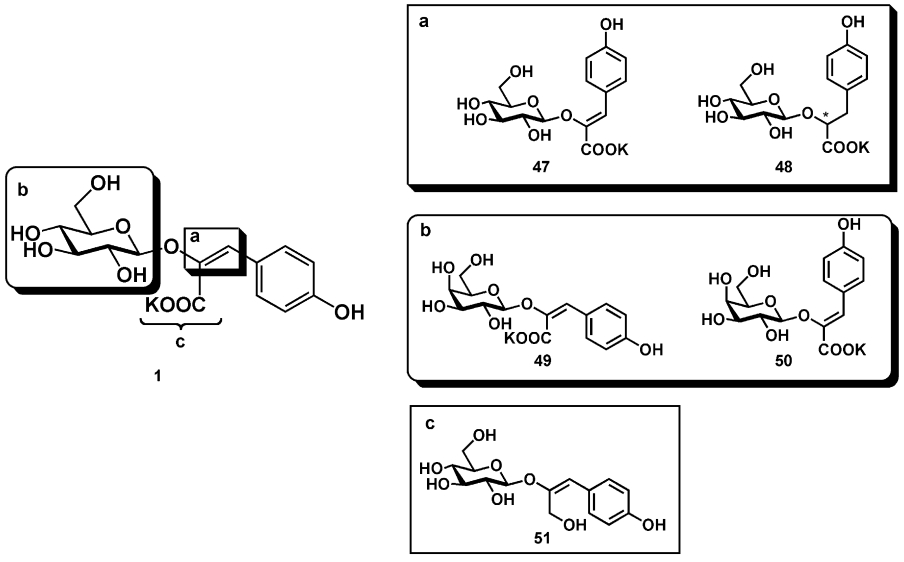


Figure 6. Substrate specificity of LOFG ((a) analogs *containing the* olefine moiety, (b) analogs containing the sugar moiety, (c) analog containing the carboxylate).

nyctinasty. The glucosylamidine ligand **6**, designed based on the structure of **1** and synthesized easily in short steps, showed strong inhibitory activity toward LOFG and was relatively effective for the selective purification of LOFG. It was also shown that the obtained LOFG strongly recognized an aglycon part of the substrate and that it exhibited high substrate specificity toward **1**. Research currently being undertaken is directed at cloning LOFG. It is important for the identification of LOFG to observe the circadian rhythm of the protein expression of this enzyme. A molecular genetic approach will reveal the mechanisms involved in the circadian rhythmic control of LOFG activity and how it was controlled by a biological clock.

3. Experimental

3.1. General procedures

NMR spectra were recorded on a Jeol JNM-AL300 [^{1}H (300 MHz) and ^{13}C (75 MHz)] using TMS in $CDCl_3$, CD_2HOD in CD_3OH (^{1}H; 3.33 ppm, ^{13}C; 49.8 ppm), or *t*-BuOH (^{1}H; 1.23 ppm, ^{13}C; 32.1 ppm) in D_2O as internal standards at various temperatures. The ESI-MS and HR ESI-MS spectra were recorded on a Bruker Esquire 3000 plus and a Bruker APEX-III, respectively. The IR spectra were recorded on a JASCO FT/IR-410. The specific rotations were measured by JASCO DIP-360 polarimeter. The HPLC purification was carried out with a Shimadzu LC-6A pump equipped with SPD-6A detector using COSMOSIL 5C_{18}-AR column (ϕ 20 × 250 mm) (Nakalai Tesque Co. Ltd). The solvents used for HPLC were available from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.45 μm pore size, 47 mm dia.) before use. Silica gel column chromatography was performed on Silica Gel 60 K070 (Katayama Chemical Co. Ltd) or Silica Gel 60N (Kanto Chemical Co. Ltd). Reversed-phase open-column chromatography was performed on Cosmosil 75C18-OPN (Nakalai Tesque Co. Ltd). TLC was performed on Silica gel F_{254} (0.25 mm or 0.5 mm, Merck) or RP-18F_{254S} (0.25 mm, Merck). Purification of the protein was performed on an ÄKTA S-100 FPLC system (GE Healthcare Co.) equipped with various columns.

3.1.1. Synthesis of *tert*-butyl trans-*p*-*O*-acetyl coumarate (9). *trans*-*p*-Coumaric acid (**8**) (5.30 g, 32.3 mmol) was dissolved in pyridine (200 mL) and Ac_2O (100 mL) was added at 0 °C under argon atmosphere. After stirring overnight at room temperature, water was added and the resulting solution was evaporated to dryness. To the residue *tert*-butyl trichloroacetimidate (24.68 g, 112.9 mmol) and THF (150 mL) was added. To the solution, $BF_3{\cdot}Et_2O$ (0.8 mL, 6.31 mmol) was added at 0 °C under argon atmosphere. After stirring for 30 min, sodium hydrogen carbonate (0.73 g) was added and stirred for another 10 min. The reaction mixture was filtered and concentrated under vacuum. The residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 5:1 to give **9** (6.01 g, 71%) as a colorless powder.

^{1}H NMR (300 MHz, $CDCl_3$, rt): 7.55 (1H, d, J = 15.6 Hz), 7.51 (2H, d, J = 8.4 Hz), 7.10 (2H, d, J = 8.4 Hz), 6.32 (1H, d, J = 15.6 Hz), 2.30 (3H, s), 1.53 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 169.1, 166.2, 151.8, 142.4, 132.4, 129.0, 122.0, 120.3, 80.6, 28.2, 21.1 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 285.1098, $C_{15}H_{18}NaO_4^+$ requires *m*/*z* 285.1097; IR (film) *v*: 2978, 2934, 1769, 1709, 1638, 1508, 1369 cm^{-1}.

3.1.2. Synthesis of *tert*-butyl 2,3-dihydroxy-3-(*p*-hydroxyphenyl)propionate (10). To a solution of **9** (13.05 g, 49.8 mmol) and *N*-methylmorpholine *N*-oxide (12.82 g, 109.6 mmol) in H_2O/acetonitrile = 1:2 (450 mL), OsO_4 dissolved in *tert*-butanol (125 mg/25 mL) was added at 0 °C under argon atmosphere. After stirring for 18 h, saturated Na_2SO_3 aq (30 mL) was added. The organic layer was separated and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried over sodium sulfate and concentrated under vacuum. The residue was dissolved in MeOH (270 mL) and sodium methoxide (1.10 g, 20.4 mmol) was added and stirred for 11 h under argon atmosphere. The reaction mixture was neutralized by Amberlite-IR120B (H^+) and filtered. Evaporation of the filtrate gave **10** (13.1 g, quant.) as a colorless oil.

^{1}H NMR (300 MHz, CD_3OD, rt): 7.19 (2H, d, J = 8.4 Hz), 7.74 (2H, d, J = 8.4 Hz), 4.68 (1H, d, J = 5.7 Hz), 4.08 (1H, d, J = 5.7 Hz), 1.31 (9H, s) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 173.2, 158.2, 132.8, 129.5, 115.8, 82.6, 75.6, 76.5, 28.1 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 277.1047, $C_{13}H_{18}NaO_5^+$ requires *m*/*z* 277.1046; IR (film) *v*: 3410, 2979, 2935, 1720, 1616, 1599, 1518, 1371 cm^{-1}.

3.1.3. Synthesis of *tert*-butyl 2,3-dihydroxy-3-(*p*-(*tert*-butyldimethylsilyloxy)phenyl)-propionate (11). Compound **10** (64.7 mg, 0.255 mmol) was dissolved in THF (2.5 mL) and TEA (71 μL) was added. To this solution, TBSCl (43.8 mg) was added at 0 °C under argon atmosphere. After stirring for 24 h, the mixture was evaporated to dryness. The residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 1:1 to give **11** (63.1 mg, 67%) as a colorless oil.

^{1}H NMR (300 MHz, CD_3OD, rt): 7.26 (2H, d, J = 8.4 Hz), 6.80 (2H, d, J = 8.4 Hz), 4.71 (1H, d, J = 5.7 Hz), 4.09 (1H, d, J = 5.7 Hz), 1.32 (9H, s), 0.98 (9H, s), 0.18 (6H, s) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 165.9, 156.5, 135.0, 129.5, 120.7, 82.6, 77.4, 76.3, 28.1, 26.1, 19.0, −4.3 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 391.1913, $C_{19}H_{32}NaO_5Si^+$ requires *m*/*z* 391.1911; IR (film) *v*: 3369, 1695, 1608, 1508 cm^{-1}.

3.1.4. Synthesis of *tert*-butyl 3-(*p*-(*tert*-butyldimethylsilyloxy)phenyl)-3-hydroxy-2-(*p*-nitrobenzenesulfonyl)propionate (12). To a stirred solution of **11** (520.5 mg, 1.41 mmol) and TEA (0.4 mL, 2.87 mmol) in dichloromethane (15 mL), *p*-nitrobenzenesulfonyl chloride (347.3 mg, 1.57 mmol) was added at 0 °C under argon atmosphere. After stirring for 3 h, the reaction mixture was concentrated and the residue was purified by silica

gel column chromatography with *n*-hexane/EtOAc = 3:1 to give **12** (621.8 mg, 80%) as a yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$, rt): 8.30 (2H, d, J = 9.0 Hz), 7.92 (2H, d, J = 9.0 Hz), 7.13 (2H, d, J = 8.4 Hz), 6.71 (2H, d, J = 8.4 Hz), 5.10 (1H, dd, J = 4.8, 5.4), 4.91 (1H, d, J = 4.8 Hz), 2.81 (1H, d, J = 5.4 Hz, –OH), 1.38 (9H, s), 1.01 (9H, s), 0.20 (6H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 165.3, 156.1, 150.4, 141.6, 130.0, 129.1, 127.7, 124.1, 120.0, 83.9, 82.9, 73.2, 27.6, 25.5, 18.1, −4.57, −4.60 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 576.1691, $C_{25}H_{35}NNaO_9SSi^+$ requires *m*/*z* 576.1694; IR (film) *v*: 3449, 1736, 1608, 1535 cm^{-1}.

3.1.5. Synthesis of *tert*-butyl 2-azido-3-(*p*-(*tert*-butyldimethylsilyloxy)phenyl)-3-hydroxypropionate (13). To a solution of **12** (507.6 mg, 0.918 mmol) in DMF (6 mL), sodium azide (313.8 mg, 4.83 mmol) was added and stirred at 50 °C under argon atmosphere. After 16.5 h, water was added to this solution and the mixture was extracted by *n*-hexane/EtOAc = 1:1. The organic layer was washed with brine and dried over absolute sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 6:1 to give **13** (146.3 g, 41%) as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$, rt): 7.25 (2H, d, J = 8.4 Hz), 6.83 (2H, d, J = 8.4 Hz), 4.92 (1H, dd, J = 4.8, 6.6 Hz), 3.97 (2H, d, J = 6.6 Hz), 3.01 (1H, d, J = 4.8 Hz, –OH), 1.45 (9H, s), 0.98 (9H, s), 0.19 (6H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 168.0, 155.9, 131.8, 127.9, 120.1, 83.6, 73.7, 67.1, 27.9, 25.6, 18.2, −4.5 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 516.1975, $C_{19}H_{31}N_3NaO_4Si^+$ requires *m*/*z* 416.1976; IR (film) *v*: 3449, 2931, 2960, 2110, 1735, 1608, 1512 cm^{-1}.

3.1.6. Synthesis of *tert*-butyl 2-azido-3-(*p*-(*tert*-butyldimethylsilyloxy)phenyl)-3-fluoropropionate (14). To a stirred solution of **13** (106.8 mg, 0.27 mmol) in dichloromethane (5 mL), DAST (72 μL, 0.55 mmol) was added at 0 °C. After stirring for 5 min, water was added and the organic layer was separated. The aqueous layer was extracted by $CHCl_3$. The combined organic layer was washed with brine and dried over absolute sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 10:1 to give colorless oil **14** (56.7 mg, 53%).

^{1}H NMR (300 MHz, $CDCl_3$, rt): 7.26 (2H, d, J = 8.4 Hz), 6.86 (2H, d, J = 8.4 Hz), 5.75 (1H, dd, J = 5.1, 45.9 Hz), 3.94 (1H, dd, J = 5.1, 22.5 Hz), 1.41 (9H, s), 0.98 (9H, s), 0.20 (6H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 166.0, 156.7, 127.8 (d, J = 6.2 Hz, C-F), 127.5 (d, J = 19.7 Hz, C-F), 120.2, 93.5 (d, J = 178.8 Hz, C-F), 83.7, 66.1 (d, J = 66.6 Hz, C-F), 27.8, 25.6, 18.2, −4.5 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 418.1935, $C_{19}H_{30}FN_3NaO_3Si^+$ requires *m*/*z* 418.1933; IR (film) *v*: 2932, 2860, 2114, 1744, 1610, 1514 cm^{-1}.

3.1.7. Synthesis of *tert*-butyl (*Z*)-2-(*N*,*N*-glucoamidine)-3-(*p*-hydroxyphenyl)acrylate (17). To a stirred solution of **14** (232.5 mg, 0.587 mmol) in MeOH (6 mL) 10% Pd–C was added and stirred for 12 h under hydrogen atmosphere. The reaction mixture was filtered on Celite and the filtrate was concentrated to dryness to give crude **15**. Crude **15** was then dissolved in DMF (5 mL) and **7** (12.6 mg, 0.065 mmol) was added to this solution. To this mixture, NBS (36.5 mg, 0.21 mmol) was added at 0 °C under argon atmosphere. After 1 h the reaction mixture was dried and the residue was purified by silica gel column chromatography with $CHCl_3$/MeOH = 9:1 followed by elution with $CHCl_3$/MeOH = 3:1 to give **16** (13.2 mg, 40%) and **17** (13.9 mg, 54%) as a colorless oil. The obtained **16** (13.2 mg, 0.025 mmol) was then stirred with TEA (5 μL, 0.034 mmol) in DMF (0.2 mL) for 1 h to give additional **17** (9.9 mg, total 94%).

Compound **16**: ^{1}H NMR (300 MHz, CD_3OD, rt): 7.39 (2H, d, J = 7.6 Hz), 6.92 (2H, d, J = 7.6 Hz), 5.85 (1H, dd, J = 6.0, 45.6 Hz), 6.14 (1H, dd, J = 6.0, 18.6 Hz), 4.22 (1H, d, J = 9.0 Hz), 3.79 (1H, dd, J = 3.3, 11.1 Hz), 3.68 (1H, dd, J = 7.2, 8.7 Hz), 3.62 (1H, dd, J = 8.7, 9.0 Hz), 3.60 (1H, dd, J = 4.2, 11.1 Hz), 3.44 (1H, ddd, J = 3.3, 4.2, 7.2 Hz), 1.34 (9H, s), 1.00 (9H, s), 0.22 (6H, s) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 166.9, 166.2 (d, J = 7.9 Hz), 158.4 (d, J = 2.4 Hz), 129.7 (d, J = 6.1 Hz), 128.8 (d, J = 20.0 Hz), 121.4, 94.4 (d, J = 178.1 Hz), 85.6, 74.0, 70.1, 69.5, 62.6, 61.7, 61.0 (d, J = 25.4 Hz), 27.9, 26.1, 19.1, −4.3 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m*/*z* 529.2740, $C_{25}H_{42}FN_2O_7Si$ requires *m*/*z* 529.2740; IR (film) *v*: 3319, 1735, 1676, 1610, 1514 cm^{-1}; $[\alpha]_D^{26}$ −15.7 (*c* 1.00, CH_3OH).

Compound **17**: ^{1}H NMR (600 MHz, CD_3OD, rt): 7.55 (1H, s), 7.51 (2H, d, J = 8.4 Hz), 6.81 (2H, d, J = 8.4 Hz), 4.30 (1H, d, J = 8.4 Hz), 3.71 (1H, dd, J = 7.2, 8.0 Hz), 3.69 (1H, dd, J = 3.0, 11.4 Hz), 3.62 (1H, dd, J = 8.0, 8.4 Hz), 3.55 (1H, dd, J = 4.2, 11.4 Hz), 3.24 (1H, ddd, J = 3.0, 4.2, 7.2 Hz), 1.54 (9H, s) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 166.6, 163.9, 162.0, 140.4, 133.9, 124.5, 120.9, 117.1, 83.7, 74.4, 70.2, 69.8, 62.4, 61.7, 28.3 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m*/*z* 395.1814, $C_{19}H_{27}N_2O_7$ requires *m*/*z* 395.1813; IR (film) *v*: 3186, 1655, 1603, 1508 cm^{-1}; $[\alpha]_D^{26}$ +11.5° (*c* 1.00, CH_3OH).

3.1.8. Synthesis of (*Z*)-2-(*N*,*N*-glucoamidine)-3-(*p*-hydroxyphenyl)acrylic acid (18). Compound **17** (1.6 mg, 4.1 μmol) was dissolved in TFA (0.5 mL) and stirred for 30 min under argon atmosphere. The reaction mixture was dried and purified by preparative TLC (RP-18, 1% AcOH, 50% MeOH aq) to give **18** (1.2 mg, 87%) as a colorless solid.

^{1}H NMR (300 MHz, CD_3OD, rt): 7.59 (1H, s), 7.43 (2H, d, J = 8.4 Hz), 6.79 (2H, d, J = 8.4 Hz), 4.32 (1H, d, J = 9.6 Hz), 3.75 (1H, dd, J = 7.2, 9.3 Hz), 3.69 (1H, dd, J = 3.0, 11.7 Hz), 3.61 (1H, dd, J = 9.3, 9.6 Hz), 3.52 (1H, dd, J = 3.6, 11.7 Hz), 3.21 (1H, ddd, J = 3.0, 3.6, 7.2 Hz) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 170.0, 165.3, 160.6, 136.4, 132.9, 125.9, 125.5, 116.8, 74.2, 70.4, 69.7, 62.3, 61.9 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m*/*z* 339.1189, $C_{15}H_{19}N_2O_7$ requires *m*/*z*

339.1187; IR (film) *v*: 3207, 1668, 1606, 1558, 1514, 1379 cm^{-1}; $[\alpha]_D^{26}$ −21.6° (*c* 0.50, CH_3OH).

3.1.9. Synthesis of (*E*)-2-(*N*,*N*-glucoamidine)-3-(*p*-hydroxyphenyl)acrylic acid (5). Compound **18** (17.2 mg, 50.9 μmol) was dissolved in MeOH (2 mL) and irradiated with UV lamp (365 nm) for 1 h. The reaction mixture was dried and purified by HPLC [Develosil-ODS-MG-5, 1% AcOH/2% MeOH aq] to give **5** (7.0 mg, 30%) and **18** (4.8 mg, 18%) as colorless solids.

Compound **5**: ^1H NMR (300 MHz, D_2O, rt): 7.40 (2H, d, *J* = 8.4 Hz), 6.88 (2H, d, *J* = 8.4 Hz), 6.73 (1H, s), 4.48 (1H, d, *J* = 8.8 Hz), 3.891 (1H, dd, *J* = 8.8, 9.5 Hz), 3.893 (1H, dd, *J* = 3.0, 12.4 Hz), 3.84 (1H, dd, *J* = 9.0, 9.5 Hz), 3.75 (1H, dd, *J* = 4.4, 12.4 Hz), 3.59 (1H, ddd, *J* = 3.0, 4.4, 9.0 Hz) ppm; ^{13}C NMR (75 MHz, D_2O, rt): 172.0, 166.0, 158.2, 133.0, 132.5, 128.0, 126.6, 117.0, 73.5, 70.3, 68.9, 61.7, 61.6 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m*/*z* 339.1188, $C_{15}H_{19}N_2O_7$ requires *m*/*z* 339.1187; IR (film) *v*: 3246, 1664, 1609, 1560, 1514, 1411 cm^{-1}; $[\alpha]_D^{26}$ −26.1 (*c* 0.40, H_2O).

3.1.10. Synthesis of 2-benzyloxy-*N*-(*p*-hydroxyphenyl) acetamide (21). To the solution of **19** (2.5 g, 22.9 mmol) and TEA (15 mL) in THF (150 mL), benzyloxyacetyl chloride (10.5 g, 56.9 mmol) was added at 0 °C under argon atmosphere. After stirring for 10 h, the resulting solution was evaporated to dryness to give crude **20**. The crude **20** was then dissolved in MeOH (150 mL), and NaOMe (3.10 g, 57.4 mmol) was added to this solution. This mixture was then stirred for 13 h at room temperature under argon atmosphere. The mixture was concentrated to 20 mL and mixed with water. This solution was then extracted by EtOAc. Organic layer was washed with brine, dried over absolute sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 2:1 to give **21** (5.90 g, quant.) as a colorless oil.

^1H NMR (300 MHz, $CDCl_3$, rt): 8.19 (1H, s), 7.46 (1H, s), 7.26 (5H, m), 7.20 (2H, d, *J* = 8.7 Hz), 6.68 (2H, d, *J* = 8.7 Hz), 4.52 (2H, s), 4.00 (2H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 168.6, 154.2, 136.7, 129.1, 129.0, 128.7, 128.4, 122.8, 116.1, 74.0, 69.6 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 280.0944, $C_{15}H_{15}NO_3$Na requires *m*/*z* 280.0942; IR (film) *v*: 3292, 3030, 2883, 1653, 1516, 1456, 1103 cm^{-1}.

3.1.11. Synthesis of 2-(*tert*-butyldimethylsilyloxy)-*N*-(*p*-(*tert*-butyldimethylsilyloxy) phenyl)acetamide (23). Compound **21** (642.5 mg 2.5 mmol) were dissolved in THF (25 mL) and 5% Pd–C was added under argon atmosphere. The mixture was stirred overnight under hydrogen atmosphere and filtered on Celite pad. The filtrate was evaporated to give **22**. Resulting **22**, imidazole (0.62 g, 9.11 mmol), and DMAP (11.0 mg, 0.09 mmol) was dissolved in DMF (10 mL). TBSCl (1.00 g, 6.62 mmol) was added at 0 °C and stirred overnight. Water was added to the resulting mixture and extracted by *n*-hexane/EtOAc = 1:1. The organic layer was washed with brine and dried over absolute sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 10:1 to give **23** (954.4 mg, quant.) as a colorless oil.

^1H NMR (300 MHz, $CDCl_3$, rt): 8.34 (1H, s, NH), 7.39 (2H, d, *J* = 9.0 Hz), 6.79 (2H, d, *J* = 9.0 Hz), 4.15 (2H, s), 0.950 (9H, s), 0.947 (9H, s), 0.15 (6H, s), 0.13 (6H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): 168.7, 152.4, 130.8, 120.9, 120.4, 63.2, 25.8, 25.6, 18.14, 18.09, −4.52, −5.57 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 418.2204, $C_{20}H_{37}NNaO_3Si_2^+$ requires *m*/*z* 418.2204; IR (film) *v*: 3395, 2932, 1638, 1508, 1236 cm^{-1}.

3.1.12. Synthesis of 2-(*tert*-butyldimethylsilyloxy)-*N*-(*p*-(*tert*-butyldimethylsilyloxy) phenyl)thioacetamide (24). Compound **23** (620.1 mg, 1.57 mmol) and Lawesson's reagent (0.50 g, 1.23 mmol) were dissolved in toluene (30 mL). This solution was stirred at 70 °C for 2 h under argon atmosphere. The reaction mixture was evaporated to 10 mL and purified by silica gel column chromatography with *n*-hexane/EtOAc = 10:1 to give **24** (559.8 mg, 87%) as a yellow solid.

^1H NMR (300 MHz, $CDCl_3$, rt): 9.97 (1H, s, NH), 7.75 (2H, d, *J* = 9.0 Hz), 6.85 (2H, d, *J* = 9.0 Hz), 4.51 (2H, s), 0.96 (9H, s), 0.95 (9H, s), 0.18 (6H, s), 0.14 (6H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 19 °C): 195.7, 154.1, 131.4, 123.5 (2C), 120.1 (2C), 70.3, 25.7 (3C), 25.6 (3C), 18.1 (2C), −4.52 (2C), −5.47 (2C) ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z*434.1977, $C_{20}H_{37}NNaO_2SSi_2^+$ requires *m*/*z* 434.1976; IR (film) *v*: 3300, 2954, 2530, 2858, 1508, 1400, 1261 cm^{-1}.

3.1.13. Synthesis of *N*-(*p*-(*tert*-butyldimethylsilyloxy) phenyl)-*N*′-(2,3,4,6-tetraacetyl-1-β-D-glucopyranosyl)-(*tert*-butyldimethylsilyloxy)methylamidine (26). Compound **24** (22.8 mg, 55.5 μmol) and $Me_3O{\cdot}BF_4$ (13.5 mg, 91.2 μmol) were dissolved in dried CH_2Cl_2. The mixture was stirred for 14 h at room temperature under argon atmosphere. To the reaction mixture, molecular sieves 4A (153.5 mg) and **25** (75.3 mg, 217 μmol) were added and the solution was stirred for 15 min. Then, mixture was cooled to 0 °C and NBS (21.4 mg, 120.2 μmol) was added. After stirring for 2 h at 0 °C, the reaction mixture was evaporated to dryness and purified by silica gel column chromatography with *n*-hexane/EtOAc = 2:1 to give **26** (35.4 mg, 89%) as a yellow oil.

^1H NMR (300 MHz, $CDCl_3$, rt): 6.71 (2H, d, *J* = 9.0 Hz), 6.57 (2H, d, *J* = 9.0 Hz), 6.26 (1H, d, *J* = 9.0 Hz, NH), 5.43 (1H, dd, *J* = 9.0, 9.0 Hz), 5.31 (1H, dd, *J* = 9.6, 9.6 Hz), 5.11 (1H, dd, *J* = 9.6, 9.9 Hz), 5.05 (1H, dd, *J* = 9.0, 9.6 Hz), 4.31 (1H, dd, *J* = 3.9, 12.6 Hz), 4.10 (1H, dd, *J* = 1.8, 12.6 Hz), 4.07 (1H, d, *J* = 15.0 Hz), 4.01 (1H, d, *J* = 15.0 Hz), 3.85 (1H, ddd, *J* = 1.8, 3.9, 9.9 Hz), 2.07 (3H, s), 2.04 (3H, s), 2.01 (3H, s), 2.00 (3H, s), 0.95 (9H, s), 0.84 (9H, s), 0.14 (6H, s), −0.02 (6H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 19 °C): 170.7, 170.2, 170.0, 169.6, 156.1, 151.1, 142.7, 121.8 (2C), 120.6 (2C), 79.2, 73.3, 73.1, 70.7, 68.5, 62.0, 59.6, 25.7 (6C), 20.8, 20.7, 20.62, 20.60, 18.23, 18.18, −4.47 (2C), −5.71 (2C) ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 747.3311, $C_{34}H_{56}N_2NaO_{11}Si_2^+$ requires *m*/*z*

747.3315; IR (film) *v*: 2932, 2858, 1753, 1655, 1499, 1366, 1227 cm^{-1}; $[\alpha]_D^{21}$ +14.3 (*c* 0.20, $CHCl_3$).

3.1.14. Synthesis of *N*-(*p*-hydroxyphenyl)-*N*′-(1-β-D-glucopyranosyl)hydroxylmethyl-amidine (6). Compound **26** (8.6 mg, 11.9 μmol) was dissolved in methanol (1 mL) and acetyl chloride (50 μL) was added at 0 °C under argon atmosphere. The reaction mixture was stirred for 24 h at room temperature and evaporated to dryness. The residue was purified by HPLC equipped with Cosmosil 5C18AR column (Nacalai Co.) and 0.1% TFA/5% MeOHaq. as an eluent to give **6** (4.3 mg, quant.) as a mixture of **6a** and **6b**.

Major isomer in D_2O: ^1H NMR (300 MHz, D_2O, 40 °C): 7.34 (2H, d, *J* = 8.7 Hz), 7.04 (2H, d, *J* = 8.7 Hz), 4.86 (2H, s), 4.76 (1H, d, *J* = 8.7 Hz), 3.89 (1H, dd, *J* = 1.8, 12.6 Hz), 3.71 (1H, dd, *J* = 4.8, 12.6 Hz), 3.55 (1H, ddd, *J* = 1.8, 4.8, 9.0 Hz), 3.54 (1H, dd, *J* = 9.0, 9.0 Hz), 3.41 (1H, dd, *J* = 8.7, 9.0 Hz), 3.39 (1H, dd, *J* = 9.0, 9.0 Hz) ppm; ^{13}C NMR (75 MHz, D_2O, 40 °C) ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m*/*z* 329.1341, $C_{14}H_{21}N_2O_7^+$ requires *m*/*z* 329.1343; IR (film) *v*: 3261, 1654, 1516, 1439, 1202 cm^{-1}.

3.1.15. Synthesis of *tert*-butyl 3-(*p*-(2-(2-(*tert*-butoxycarbonylsulfanyl)ethoxy)ethoxy)-phenyl)-2,3-dihydroxypropionate (29). To a stirred solution of **10** (4.91 g, 19.3 mmol) and 2-(2-(*tert*-butoxycarbonylsulfanyl)ethoxy)ethyl iodide (7.04 g, 21.2 mmol) in DMF, sodium hydride in oil (1.07 g) was added at 0 °C under argon atmosphere. After stirring for 15 h, water (1200 mL) was added and the mixture was extracted with *n*-hexane/EtOAc = 1:1. The organic layer was washed with brine and dried over absolute sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 1:1 to give **29** (3.88 g, 44%) as an orange oil.

^1H NMR (300 MHz, $CDCl_3$, rt): 7.31 (2H, d, *J* = 8.7 Hz), 6.90 (2H, d, *J* = 8.7 Hz), 4.84 (1H, d, *J* = 3.6 Hz), 4.20 (1H, d, *J* = 3.6 Hz), 4.10 (2H, t, *J* = 4.8 Hz), 3.83 (2H, t, *J* = 4.8 Hz), 3.71 (2H, t, *J* = 6.6 Hz), 3.01 (2H, t, *J* = 6.6 Hz), 1.49 (9H, s), 1.43 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 171.9, 168.9, 158.4, 132.5, 127.7, 114.4, 84.8, 83.1, 74.7, 74.4, 70.2, 69.3, 67.3, 30.3, 28.1, 27.8 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m*/*z* 481.1867, $C_{22}H_{35}O_8S^+$ requires *m*/*z* 481.1868; IR (film) *v*: 3473, 2979, 2933, 2875, 1716, 1703, 1612, 1514, 1369 cm^{-1}.

3.1.16. Synthesis of *tert*-butyl 3-(*p*-(2-(2-(*tert*-butoxycarbonylsulfanyl)ethoxy)ethoxy)-phenyl)-3-hydroxy-2-(*p*-nitrobenzenesulfonyl)propionate (30). To a stirred solution of **29** (3.88 g, 8.47 mmol) and TEA (2.4 mL, 17.2 mmol) in dichloromethane, *p*-nitrobenzenesulfonyl chloride (1.88 g, 8.47 mmol) was added at 0 °C under argon atmosphere. After stirring for an hour, the reaction mixture was concentrated and the residue was purified by silica gel column chromatography with $CHCl_3$/EtOAc = 7:1 to give **30** (5.09 g, 93%) as a yellow solid.

^1H NMR (300 MHz, $CDCl_3$, 20 °C): 8.22 (2H, d, *J* = 9.0 Hz), 7.85 (2H, d, *J* = 9.0 Hz), 7.12 (2H, d, *J* = 8.7 Hz), 6.73 (2H, d, *J* = 8.7 Hz), 5.09 (1H, m), 4.86 (1H, d, *J* = 4.2 Hz), 4.04 (2H, t, *J* = 4.5 Hz), 3.83 (2H, t, *J* = 4.5 Hz), 3.73 (2H, t, *J* = 6.6 Hz), 3.03 (2H, t, *J* = 6.6 Hz), 2.63 (1H, d, *J* = 4.2 Hz, –OH), 1.49 (9H, s), 1.38 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): 169.0, 165.3, 159.0, 150.4, 141.7, 129.7, 129.1, 127.6, 124.0, 114.4, 84.9, 84.0, 82.9, 73.1, 70.3, 69.2, 67.4, 30.4, 28.2, 27.7 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 666.1650, $C_{28}H_{37}NNaO_{12}S_2^+$ requires *m*/*z* 666.1649; IR (film) *v*: 3508, 2982, 2936, 2876, 1757, 1716, 1699, 1610, 1533, 1514, 1369, 1350 cm^{-1}.

3.1.17. Synthesis of *tert*-butyl 2-azido-3-(*p*-(2-(2-(*tert*-butoxycarbonylsulfanyl)ethoxy)-ethoxy)phenyl)-3-hydroxypropionate (31). To a stirred solution of **30** (5.09 g, 7.92 mmol) in DMF (80 mL), sodium azide (2.60 g, 40.0mmol) was added and heated to 60 °C. After 15 h, water (550 mL) was added and extracted with EtOAc. The organic layer was washed with brine and dried over absolute sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 4:1 to give **31** (2.37 g, 61%) as a yellow solid.

^1H NMR (300 MHz, $CDCl_3$, rt): 7.30 (2H, d, *J* = 9.0 Hz), 6.91 (2H, d, *J* = 9.0 Hz), 4.92 (1H, dd, *J* = 4.8, 6.6 Hz), 4.11 (2H, t, *J* = 4.8 Hz), 3.97 (1H, d, *J* = 6.6 Hz), 3.83 (2H, t, *J* = 4.8 Hz), 3.71 (2H, t, *J* = 6.6 Hz), 3.04 (1H, d, *J* = 4.8 Hz, –OH), 3.01 (2H, t, *J* = 6.6 Hz), 1.49 (9H, s), 1.46 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): 169.0, 167.9, 158.8, 131.5, 127.9, 114.5, 84.8, 83.6, 73.5, 70.3, 69.3, 67.3, 67.0, 30.3, 28.1, 27.9 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m*/*z* 506.1932, $C_{22}H_{33}N_3NaO_7S^+$ requires *m*/*z* 506.1931; IR (film) *v*: 3447, 2979, 2936, 2876, 2110, 1719, 1701, 1612, 1514, 1369 cm^{-1}.

3.1.18. Synthesis of *tert*-butyl 2-azido-3-(*p*-(2-(2-(*tert*-butoxycarbonylsulfanyl)ethoxy)-ethoxy)phenyl)-3-fluoropropionate (32). To a stirred solution of **31** (3.10 g, 6.42 mmol) in dichloromethane (120 mL), DAST (1.7 mL, 128.8 mmol) was added at 0 °C. After 10 min, methanol (10 mL) was added. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/EtOAc = 6:1 to give **32** (1.88 g, 60%) as a yellow solid. Two diastereomers (**32a** and **32b**) can be separated by column chromatography.

Compound **32a**: ^1H NMR (300 MHz, $CDCl_3$, rt): 7.32 (2H, d, *J* = 8.4 Hz), 6.94 (2H, d, *J* = 8.4 Hz), 5.70 (1H, dd, *J* = 6.0, 44.4 Hz), 4.18 (1H, dd, *J* = 6.0, 10.8 Hz), 4.13 (2H, t, *J* = 4.8 Hz), 3.85 (2H, t, *J* = 4.8 Hz), 3.72 (2H, t, *J* = 6.6 Hz), 3.02 (2H, t, *J* = 6.6 Hz), 1.49 (9H, s), 1.47 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 169.0, 166.0 (d, *J* = 5.4 Hz, C-F), 159.6 (d, *J* = 1.9 Hz, C-F), 128.0 (d, *J* = 6.7 Hz, C-F), 127.1 (d, *J* = 20.6 Hz, C-F), 114.6, 92.3 (d, *J* = 177.5 Hz, C-F), 84.9, 83.8, 70.3, 69.2, 67.4, 65.8 (d, *J* = 29.0 Hz, C-F), 30.4, 28.2, 27.9 ppm; HR ESI MS (positive): $[M+Na]^+$. Found *m*/*z* 508.1890, $C_{22}H_{32}FN_3NaO_6S^+$ requires *m*/*z*

508.1888; IR (film) *v*: 2979, 2936, 2876, 2114, 1718, 1701, 1614, 1516, 1369 cm^{-1}.

Compound **32b**: ^1H NMR (300 MHz, $CDCl_3$, rt): 7.31 (2H, d, J = 8.4 Hz), 6.95 (2H, d, J = 8.4 Hz), 5.79 (1H, dd, J = 4.8, 45.3 Hz), 4.13 (2H, t, J = 4.8 Hz), 3.93 (1H, dd, J = 4.8, 23.4 Hz), 3.85 (2H, t, J = 4.8 Hz), 3.72 (2H, t, J = 6.6 Hz), 3.02 (2H, t, J = 6.6 Hz), 1.49 (9H, s), 1.43 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 169.0, 166.1 (d, J = 6.7 Hz, C-F), 159.5 (d, J = 1.8 Hz, C-F), 127.7 (d, J = 6.6 Hz, C-F), 127.5 (d, J = 20.6 Hz, C-F), 114.7, 93.5 (d, J = 178.7 Hz, C-F), 84.9, 83.9, 70.4, 69.3, 67.4, 66.6 (d, J = 23.6 Hz, C-F), 30.4, 28.2, 27.8 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m/z* 508.1888, $C_{22}H_{32}FN_3NaO_6S^+$ requires *m/z* 508.1888; IR (film) *v*: 2979, 2936, 2876, 2116, 1717, 1699, 1614, 1516, 1369 cm^{-1}.

3.1.19. Synthesis of *tert*-butyl 2-amino-3-(*p*-(2-(2-(*tert*-butoxycarbonylsulfanyl)ethoxy)-ethoxy)phenyl)-3-fluoro-propionate (33). To a solution of **32** (1.06 g, 2.18 mmol) in MeOH (44 mL), 10% Pd–C was added and stirred for 12 h under hydrogen atmosphere. The reaction mixture was filtered on Celite and the filtrate was concentrated to give **33** (1.06 g, quant.) as a slightly yellow oil. Two diastereomers (**33a** and **33b**) were separated by column chromatography with *n*-hexane/EtOAc = 6:1.

Compond **33a**: ^1H NMR (300 MHz, $CDCl_3$, rt): 7.28 (2H, d, J = 8.4 Hz), 6.93 (2H, d, J = 8.4 Hz), 5.56 (1H, dd, J = 6.0, 45.0 Hz), 4.12 (2H, t, J = 4.8 Hz), 3.85 (1H, dd, J = 4.8, 11.3 Hz), 3.84 (2H, t, J = 4.8 Hz), 3.72 (2H, t, J = 6.6 Hz), 3.02 (2H, t, J = 6.6 Hz), 1.49 (9H, s), 1.45 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 171.0 (d, J = 6.7 Hz, C-F), 169.0, 159.2 (d, J = 1.8 Hz, C-F), 128.1 (d, J = 20.6 Hz, C-F), 127.9 (d, J = 6.6 Hz, C-F), 114.5, 94.4 (d, J = 175.7 Hz, C-F), 84.9, 82.0, 70.3, 69.3, 67.4, 59.7 (d, J = 26.6 Hz, C-F), 30.4, 28.2, 28.0 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m/z* 482.1982, $C_{22}H_{34}FNNaO_6S^+$ requires *m/z* 482.1983; IR (film) *v*: 3393, 2979, 2933, 2874, 1719, 1701, 1614, 1516, 1369 cm^{-1}.

Compound **33b**: ^1H NMR (300 MHz, $CDCl_3$, rt): 7.28 (2H, d, J = 8.4 Hz), 6.94 (2H, d, J = 8.4 Hz), 5.63 (1H, dd, J = 4.5, 46.2 Hz), 4.12 (2H, t, J = 4.8 Hz), 3.84 (2H, t, J = 4.8 Hz), 3.72 (2H, t, J = 6.6 Hz), 3.67 (1H, dd, J = 4.5, 23.1 Hz), 3.02 (2H, t, J = 6.6 Hz), 1.49 (9H, s), 1.40 (9H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 170.8 (d, J = 5.4 Hz, C-F), 169.0, 159.0 (d, J = 1.8 Hz, C-F), 129.0 (d, J = 21.2 Hz, C-F), 127.4 (d, J = 7.3 Hz, C-F), 114.5, 94.9 (d, J = 175.1 Hz, C-F), 84.9, 82.0, 70.3, 69.3, 67.4, 60.5 (d, J = 24.2 Hz, C-F), 30.4, 28.2, 27.9 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m/z* 482.1982, $C_{22}H_{34}FNNaO_6S^+$ requires *m/z* 482.1983; IR (film) *v*: 3396, 2979, 2933, 2876, 1720, 1703, 1614, 1516, 1369 cm^{-1}.

3.1.20. Synthesis of *tert*-butyl (*Z*)-2-(*N*,*N*-glucoamidine)-3-(*p*-(2-(2-mercaptoethoxy)-ethoxy)phenyl)acrylate (34). To a stirred solution of **33** (14.2 mg, 31.0 μmol) and **7** (1.2 mg, 6.2 μmol) in MeOH (150 μL), NBS (3.5 mg, 20.0 μmol) was added at 0 °C under argon atmosphere. After 2 h, the reaction mixture was evaporated to dryness and the residue was dissolved in THF (0.2 mL). To this solution, TEA (10 μL) was added and the mixture was stirred for 10 min. After evaporation, the residue was purified by preparative TLC with $CHCl_3$/MeOH/AcOH = 60:10:1 to give **34** (4.3 mg, 23%) as a yellow solid.

^1H NMR (300 MHz, CD_3OD, rt): 7.78 (1H, s), 7.62 (2H, d, J = 8.7 Hz), 7.02 (2H, d, J = 8.7 Hz), 4.41 (1H, d, J = 9.6 Hz), 4.17 (2H, t, J = 4.5 Hz), 3.86-3.80 (3H, m), 3.66 (2H, t, J = 6.6 Hz), 3.66–3.60 (3H, m), 3.31–3.29 (1H, m), 2.99 (2H, t, J = 6.6 Hz), 1.56 (9H, s), 1.47 (9H, s) ppm; ^{13}C NMR (75 MHz, CD_3OD, 18 °C): 170.5, 166.9, 163.6, 162.7, 140.7, 133.7, 125.8, 121.3, 116.2, 85.8, 83.9, 74.3, 71.3, 70.3, 70.0, 69.8, 68.8, 62.6, 61.6, 31.2, 28.4, 28.3 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m/z* 599.2632, $C_{28}H_{43}N_2O_{10}S^+$ requires *m/z* 599.2633; IR (film) *v*: 3307, 1701, 1661, 1601, 1510, 1369 cm^{-1}; $[\alpha]_D^{19}$ +7.1 (*c* 0.90, CH_3OH).

3.1.21. Synthesis of (*Z*)-2-(*N*,*N*-glucoamidine)-3-(*p*-(2-(2-mercaptoethoxy)ethoxy)-phenyl)acrylic acid (35). Compound **34** (285.9 mg, 0.463 mmol) was dissolved in TFA (6.0 mL) and stirred for an hour at 0 °C under argon atmosphere. The reaction mixture was dried and purified by column chromatography using Cosmosil 75C_{18}-OPN (Nakalai Tesque Co., Ltd) with 0.5% TFA/20% MeOH aq. to give **35** (119.5 mg, 58%) as a yellow solid.

^1H NMR (300 MHz, CD_3OD, rt): 7.65 (1H, s), 7.52 (2H, d, J = 8.7 Hz), 6.98 (2H, d, J = 8.7 Hz), 4.36 (1H, d, J = 9.6 Hz), 4.16 (2H, t, J = 4.8 Hz), 3.82 (2H, t, J = 4.8 Hz), 3.76 (1H, dd, J = 7.2, 9.6 Hz), 3.70 (1H, dd, J = 3.0, 12.0 Hz), 3.65 (2H, t, J = 6.6 Hz), 3.62 (1H, dd, J = 9.6, 9.6 Hz), 3.53 (1H, dd, J = 4.2, 12.0 Hz), 3.22 (1H, m), 2.67 (2H, t, J = 6.6 Hz) ppm; ^{13}C NMR (75 MHz, D_2O, 23 °C): 171.0, 164.9, 161.3, 138.1, 133.0, 127.0, 125.8, 116.8, 73.9, 73.4, 70.2, 69.1, 68.8, 61.6, 61.5, 24.7 ppm; HR ESI MS (positive): $[M + H]^+$. Found *m/z* 443.1481, $C_{19}H_{27}N_2O_8S^+$ requires *m/z* 443.1483; IR (film) *v*: 3234, 1666, 1603, 1510, 1379 cm^{-1}; $[\alpha]_D^{19}$ +1.1 (*c* 0.50, H_2O).

3.1.22. Synthesis of (*E*)-2-(*N*,*N*-glucoamidine)-3-(*p*-(2-(2-mercaptoethoxy)ethoxy)-phenyl)acrylic acid (36). Compound **35** (17.2 mg, 50.9 μmol) was dissolved in H_2O (2 mL) and irradiated with UV lamp (312 nm) for 1 h. The reaction mixture was dried and purified by HPLC [Develosil-ODS-MG-5, 0.5% TFA/35% MeOH aq] to give **35** (3.5 mg, 20%) and **36** (4.8 mg, 28%) as yellow solids.

Compound **36**: ^1H NMR (300 MHz, CD_3OD, rt): 7.54 (2H, d, J = 8.7 Hz), 6.90 (2H, d, J = 8.7 Hz), 7.22 (1H, s), 4.29 (1H, d, J = 8.4 Hz), 4.13 (2H, t, J = 4.5 Hz), 3.86 (1H, dd, J = 2.4, 11.4 Hz), 3.81 (2H, t, J = 4.5 Hz), 3.71–3.65 (3H, m), 3.65 (2H, t, J = 6.6 Hz), 3.44 (1H, m), 2.66 (2H, t, J = 6.6 Hz) ppm; ^{13}C NMR (75 MHz, CD_3OD, 25 °C): 167.4, 165.4, 162.1, 144.4, 134.3, 130.9, 126.3, 115.1, 74.24, 74.17, 70.3, 70.1, 69.6, 68.7, 62.6, 61.5, 24.7 ppm; HR ESI-

MS (positive): $[M+H]^+$. Found *m/z* 443.1481, $C_{19}H_{27}N_2O_8S^+$ requires *m/z* 443.1483; IR (film) *v*: 3250, 1655, 1574, 1512, 1423 cm^{-1}; $[\alpha]_D^{19}$ −30.4 (*c* 0.10, MeOH).

3.1.23. Preparation of gluconoamidine-type affinity gel (27). To a gel **37** (2 mL) which was prepared from Toyopearl AF-Amino-650M (Tosoh Co. Ltd), **36** (488.9 mg, 1.11 mmol) dissolved in 0.1 M sodium acetate buffer (pH 5.5) was added and shaken overnight at 4 °C. The resulting gel was washed with 0.1 M acetate buffer (pH 5.5) to give gluconoamidine-type affinity gel (**27**).

3.1.24. Synthesis of *p*-(2-azidoethoxyethoxy)-*N*-(2-benzyloxyacetyl)aniline (39). Compound **21** (2.05 g, 7.98 mmol) and **38** (2.74 g, 9.61 mmol) were dissolved in DMF and cooled to 0 °C. To this solution NaH in oil (389.4 mg) was added under argon atmosphere. After stirring for 3 h, the reaction mixture was heated to 30 °C and stirred for another 3 h. To the resulting solution, water was added and the mixture was extracted by *n*-hexane/EtOAc = 1:1. The organic layer was washed with brine and dried over sodium sulfate. After evaporation, the residue was purified by silica gel column chromatography with *n*-hexane/Acetone = 3:1 to give **39** (2.02 g, 68%) as a yellow oil.

^1H NMR (300 MHz, $CDCl_3$, rt): 8.23 (1H, s), 7.45 (2H, d, *J* = 8.9 Hz), 7.38 (5H, m), 6.88 (2H, d, *J* = 8.9 Hz), 4.64 (2H, s), 4.11 (2H, t, *J* = 4.6 Hz), 4.09 (2H, s), 3.85 (2H, t, *J* = 4.6 Hz), 3.74 (2H, t, *J* = 5.0 Hz), 3.41 (2H, t, *J* = 5.0 Hz) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 167.6, 155.9, 136.9, 130.8, 129.0, 128.7, 128.4, 121.8, 115.3, 74.0, 70.5, 70.0, 69.9, 68.0, 51.0 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m/z* 393.1533, $C_{19}H_{22}N_4O_4Na$ requires *m/z* 393.1534; IR (film) *v*: 3385, 3036, 2870, 2112, 1676, 1508, 1454, 1111 cm^{-1}.

3.1.25. Synthesis of *p*-(2-azidoethoxyethoxy)-*N*-(2-benzyloxythioacetyl)aniline (40). Compound **39** (2.02 g, 5.46 mmol) and Lawesson's reagent (1.65 g, 4.08 mmol) were dissolved in toluene (100 mL) and stirred at 70 °C for an hour under argon atmosphere. The reaction mixture was concentrated to 30 mL and then purified by silica gel column chromatography with *n*-hexane/acetone = 4:1 to give **40** (1.22 g, 58%) as a yellow oil.

^1H NMR (300 MHz, $CDCl_3$, rt): 9.79 (1H, s), 7.68 (2H, d, *J* = 8.7 Hz), 7.38 (5H, m), 6.94 (2H, d, *J* = 8.7 Hz), 4.68 (2H, s), 4.48 (2H, s), 4.15 (2H, t, *J* = 4.8 Hz), 3.87 (2H, t, *J* = 4.8 Hz), 3.75 (2H, t, *J* = 4.9 Hz), 3.42 (2H, t, *J* = 4.9 Hz) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, rt): 195.9, 157.6, 136.8, 131.2, 129.1, 128.8, 128.5, 125.0, 115.1, 76.9, 73.8, 70.6, 70.0, 68.0, 51.0 ppm; HR ESI-MS(positive): $[M+Na]^+$. Found *m/z* 409.1305, $C_{19}H_{22}N_4O_3SNa$ requires *m/z* 409.1307; IR (film) *v*: 3308, 2924, 2106, 1516, 1251, 1128, 1067 cm^{-1}.

3.1.26. Synthesis of *N*-(*p*-(2-azidoethoxyethoxy)phenyl)-*N′*-(2,3,4,6-tetraacetyl-1-β-D-glucopyranosyl)benzyloxymethylamidine (42). Compound **40** (60.8 mg, 0.158 mmol) and $Me_3O{\cdot}BF_4$ (36.2 mg, 0.245 mmol) were dissolved in dried CH_2Cl_2 (5 mL). The mixture was stirred for 2 h at room temperature under argon atmosphere. To the reaction mixture, molecular sieves 4A (409 mg) and **41** (214.1 mg, 0.617 mmol) were added and stirred for 15 min. Then, the mixture was cooled to 0 °C and then NBS (58.9 mg, 0.331 mmol) was added. After stirring for 10 min at 0 °C, the reaction mixture was evaporated to dryness and purified by silica gel column chromatography with toluene/acetone = 4:1 to give **42** (61.6 mg, 56%).

^1H NMR (300 MHz, $CDCl_3$, rt): 7.35–7.27 (5H, m), 6.81 (2H, d, *J* = 8.7 Hz), 6.63 (2H, d, *J* = 8.7 Hz), 6.21 (1H, d, *J* = 8.3 Hz, NH), 5.49 (1H, dd, *J* = 8.3, 8.7 Hz), 5.34 (1H, dd, *J* = 9.6, 9.6 Hz), 5.12 (1H, dd, *J* = 9.6, 9.6 Hz), 5.07 (1H, dd, *J* = 8.7, 9.6 Hz), 4.42 (2H, s), 4.32 (1H, dd, *J* = 3.9, 12.3 Hz), 4.13 (1H, dd, *J* = 1.8, 12.3 Hz), 4.10 (2H, t, *J* = 4.5 Hz), 4.01 (1H, d, *J* = 14.4 Hz), 3.95 (1H, d, *J* = 14.4 Hz), 3.86 (1H, ddd, *J* = 1.8, 3.9, 9.6 Hz), 3.85 (2H, t, *J* = 4.5 Hz), 3.75 (2H, t, *J* = 4.8 Hz), 3.42 (2H, t, *J* = 4.8 Hz), 2.08 (3H, s), 2.05 (3H, s), 2.032 (3H, s), 2.026 (3H, s) ppm; ^{13}C NMR (75 MHz, acetone-d_6, 19 °C): 170.7, 170.6, 170.2, 170.0, 155.3, 155.1, 143.6, 138.4, 129.1, 128.6, 128.5, 122.7, 115.6, 79.1, 73.8, 73.7, 73.3, 71.8, 70.8, 70.3, 69.5, 68.4, 66.1, 62.8, 51.3, 20.634, 20.626, 20.59, 20.53 ppm; HR ESI-MS (positive): $[M+Na]^+$. Found *m/z* 722.2646, $C_{33}H_{41}N_5NaO_{12}^+$ requires *m/z* 722.2644; IR (film) *v*: 2110, 1751, 1655, 1502, 1367, 1228 cm^{-1}; $[\alpha]_D^{21}$ +9.8 (*c* 1.00, $CHCl_3$).

3.1.27. Synthesis of *N*-(*p*-(2-azidoethoxyethoxy)phenyl)-*N′*-(1-β-D-glucopyranosyl)-benzyloxymethylamidine (44). Compound **42** (64.9 mg, 92.9 μmol) was dissolved in methanol (4 mL) and acetyl chloride (200 μL) was added at 0 °C under argon atmosphere. The reaction mixture was stirred for 24 h at room temperature and evaporated to give crude **43**. The crude **43** and $Pd(OH)_2$ (10 mg) were dissolved in THF/MeOH = 1:1 (5 mL) and stirred for 3 h under hydrogen atmosphere. Suspension was then filtered on Celite pad and the filtrate was evaporated to dryness. The residue was purified by HPLC equipped with Cosmosil 5C18AR column (Nacalai Co.) with 0.1% TFA/5% methanol aq as an eluent to give **44** (29.3 mg, 81%).

Compound **44**: ^1H NMR (300 MHz, D_2O, 40 °C): 7.34 (2H, d, *J* = 8.7 Hz), 7.15 (2H, d, *J* = 8.7 Hz), 4.85 (2H, s), 4.77 (1H, d, *J* = 8.4 Hz), 4.27 (2H, t, *J* = 4.5 Hz), 3.94 (2H, t, *J* = 4.5 Hz), 3.87 (1H, dd, *J* = 2.4, 12.3), 3.83 (2H, t, *J* = 4.8 Hz), 3.70 (1H, dd, *J* = 5.1, 12.3 Hz), 3.55 (1H, ddd, *J* = 2.4, 5.1, 9.0 Hz), 3.53 (1H, dd, *J* = 9.0, 9.0 Hz), 3.39 (1H, dd, *J* = 8.4, 9.0 Hz), 3.37 (1H, dd, *J* = 9.0, 9.0 Hz), 3.23 (2H, t, *J* = 4.5 Hz) ppm; ^{13}C NMR (75 MHz, D_2O, 40 °C): 170.3, 160.4, 129.4, 127.0, 117.9, 83.5, 79.0, 77.5, 73.3, 70.7, 70.5, 69.1, 68.2, 62.0, 58.9, 40.7 ppm; HR ESI-MS (positive): $[M+H]^+$. Found *m/z* 416.2028, $C_{18}H_{31}N_3O_8^+$ requires *m/z* 416.2027; IR (film) *v*: 3254, 2891, 1682, 1512, 1202 cm^{-1}.

3.1.28. Preparation of glucosylamidine-type affinity gel (46). To gel **45** (2 mL) prepared from Toyopearl AF-Carboxyl-650M (Tosoh Co. Ltd), **44** (104.8 mg,

0.25 mmol) dissolved in 0.1 M Bis–Tris buffer (pH 7.0) was added and shaken overnight at 4 °C. The resulting gel was washed with water to obtain glucosylamidine-type affinity gel (**46**).

3.2. Determination of K_i values of 5, 6a, 6b, and 18 against glycosidases

K_i values of inhibitor **5**, **6a**, **6b**, and **18** were determined by Lineweaver–Burk plot. Each enzyme (Sigma Co., Ltd) was preincubated with various amounts of inhibitor at 37 °C for 15 min and then *p*-nitrophenyl glycoside was added to the mixture. The reaction was stopped by adding 0.5 M Na_2CO_3 aq Absorbance at 405 nm was measured by microplatereader Model 680 (Bio-Rad Co., Ltd) to determine quantity of resulting *p*-nitrophenol. And the similar experiments were carried out at various pHs (pH 4.5–5.5: 25 mM acetate buffer, pH 6.0: 25 mM Bis–Tris buffer) to determine the effect of pH on the inhibitory activity.

3.3. Stability of 5 and 6

Compound **5** or **6** was dissolved in buffer (pH 4.5–5.5: 25 mM acetate buffer, pH 6.0–7.0: 25 mM Bis–Tris buffer, pH 7.5–9.0: 25 mM Tris–HCl buffer) and incubated for 6 h at 4 °C. The remaining inhibitor was quantified by HPLC equipped with Develosil ODS-HG-5 column (Nomura Co.) using 0.1% TFA/2% methanol aq as an eluent.

3.4. Measurement of β-glucosidase activity using 1

Twenty-five millimolar acetate buffer (pH 4.5) containing 1 mM potassium lespedezate (**1**) and 1 mM semicarbazide hydrochloride (**48**) was preincubated at 37 °C for 15 min. β-Glucosidase in 25 mM acetate buffer (pH 4.5) was added to this solution, and the mixture was incubated at 37 °C. After 2 h, the reaction was stopped by adding 0.1 M Tris–HCl buffer (pH 9.0) and adjusting the pH to 8.0. Quantitative analysis of the resulting semicarbazone (**49**) was performed by HPLC with Develosil ODS-HG-5 (Nomura Chemical Co.) using 0.1% TFA, 20% MeOH aq as an eluent. One enzyme unit of LOFG was defined as a quantity of enzyme that hydrolyzes one micro mole of **1** in one minute under optimum pH condition at 30 °C.

3.5. Purification of LOFG from the leaves of *L. cuneata*

The purification of LOFG from *L. cuneata* was carried out according to the following procedure. In each purification step, amount of proteins were quantified with Quick Starttrade Bradford Protein Assay (Bio-Rad Co., Ltd) using BSA (20, 10, 5, 2.5, 1.25 μg/mL) as a standard.

3.5.1. Preparation of crude enzyme. Leaves of *L. cuneata*(180 g) were collected at certain time of the day and homogenized in cold acetone (−20 °C) by Warring Blender. Resulting homogenate was filtrated by miracloth under vacuo and washed with cold acetone until the filtrate became nearly colorless. The residue was dried *in vacuo* to give acetone powder (50 g). Acetone powder (50 g) was dissolved in 800 mL of 0.1 M Bis–Tris buffer (pH 7.0) containing 2 mM DTT, 5 mM EDTA, 1 M NaCl, ascorbic acid (1 g) and Polyclar-VT (8% w/v). The mixture was stirred overnight at 4 °C, and then centrifuged at 14,000*g* for 30 min. Resulting supernatant (500 mL) was collected and used as a crude enzyme.

3.5.2. Acetone precipitation. To the crude enzyme solution (500 mL), 750 mL of chilled acetone (−20 °C) was added gradually and stirred at 0 °C for 5 h. Resulting precipitate was collected by centrifugation (14,000*g* 30 min.) and suspended in 100 mL of 25 mM Bis–Tris buffer (pH 7.0). The suspension was stirred for 5 h and then centrifuged (14,000*g* 30 min.). The obtained supernatant was used for further purification.

3.5.3. Purification by Hiprep 16/10 Q XL. The abovementioned supernatant (80 mL) was applied to Hiprep 16/10 Q XL (GE Healthcare Co.) equilibrated with 25 mM Bis–Tris buffer (pH 7.0). The column was washed with the same buffer containing 0–0.3 M sodium chloride (linear gradient in 120 mL), 0.3–1.0 M sodium chloride (linear gradient in 20 mL), and 1.0 M sodium chloride (40 mL). The fractions were collected in 20 mL each and concentrated to 4 mL by ultrafiltration (Amicon ultra-15, MWC 10, 000, Millipore Co.) and desalted by Hitrap Desalting column (GE Healthcare Co.). LOFG activity and protein concentration of each fraction were analyzed by the method mentioned above.

3.5.4. Affinity purification at pH 5.0. Crude LOFG solution (4 mL) purified by HiPrep 16/10 Q XL was applied to the column ($\phi \times 100$ mm) with 2 mL of affinity gel **27** or **46**. After washing the column by 50 mM acetate buffer (pH 5.0), stepwise elution using the same buffer containing 2 M D-glucose (10 mL), 10 mM potassium lespedezate (**1**, 10 mL) and then 1 M NaCl (10 mL) was performed. The fractions were collected 5 mL each and desalted by Hitrap Desalting column, and concentrated by Amicon Ultra-4 to 2 mL.

3.5.5. Superdex 200 10/300 GL gel-filtration chromatography. Resulting LOFG solution containing **1** (0.5 mL) was applied to Superdex 200 10/30 GL (GE Healthcare bio.) equilibrated with 25 mM Acetate buffer (pH 4.5) containing 100 mM sodium chloride. Elution was performed by the same buffer at a flow late of 0.5 mL/min to give pure LOFG.

3.5.6. Reverse-phase HPLC analysis of LOFG. LOFG was analyzed by HPLC equipped with Sephasil Protein C4 5 μm ST4.6/250 (GE Healthcare Co.). The linear gradient analysis between eluent A (0.065% TFA, 2% MeCN aq.) and eluent B (0.05% TFA, MeCN) in 50 min was performed at a flow rate of 1.0 mL/min at 40 °C. LOFG was detected at 215 nm.

3.5.7. Gel-filtration HPLC analysis of LOFG. LOFG was analyzed by HPLC equipped with TSK-Gel SuperSW3000 (Tosoh Co. Ltd). The analysis was performed at a flow rate of 0.2 mL/min and 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.0) as an

eluent. The proteins were detected at 215 nm. IgG 150 kDa, BSA 68 kDa, Carbonic anhydrase (CA) 34 kDa, and cytochrome C (Cyt C) 12 kDa were used as molecular weight standards.

3.5.8. SDS–PAGE analysis of LOFG. SDS–PAGE was performed according to the method of Laemmli.[47] Precision Plus Protein Standards (Bio-Rad Inc.) were used as a marker protein. Samples (30 μL) were mixed with 6 μL of SDS sample buffer (0.35 M Tris–HCl, 10% SDS, 30% glycerol, 600 mM DTT, 0.01% BPB, pH 6.8) and heated at 95 °C for 5 min. Fifteen microliters of each mixture was applied to 10% polyacrylamide gel and electrophoresised at 200 V for 45 min. Protein bands were visualized by silver staining using Bio-Rad Silver Stain kit (Bio-Rad Inc.).

3.5.9. K_m value of LOFG. K_m value of LOFG was estimated from Liveweaver–Burk plot with various concentrations of **3** (250, 500, 750, 1000 μM) as substrates described above.

3.5.10. De novo sequenece analysis of LOFG. Purified LOFG was hydrolyzed by trypsin in H_2O and $H_2{}^{18}O$, respectively, in appropriate conditions. Then the two hydrolyzates were analyzed by MS/MS using oMALDI-Qq-TOF MS/MS QSTAR Pulsar *i* (Applied Biosystems Co., Ltd) with de novo sequence analyis software PEAKS (Infocom Co., Ltd). Comparison of obtained data gave partial sequences and the de novo sequence was obtained from the agreement between them (Table 4).

3.6. Substrate specificity of LOFG

Substrate specificity of LOFG was examined as follows: compounds **47**–**51** were hydrolyzed by LOFG, respectively. Twenty-five millimolars acetate buffer (pH 4.5) containing each substrate (1 mM) were preincubated at 37 °C. LOFG in 25 mM acetate buffer (pH 4.5) was added to this solution, and the mixture was incubated at 37 °C. After two hours, the reaction was stopped by adding 0.1 M Tris–HCl buffer (pH 9.0) and adjusting the pH to 8.0. Quantitative analysis of resulting aglycon was performed by HPLC using Develosil ODS-HG-5 (Nomura Chemical Co.) with 0.1% TFA, 20% MeOH aq.

3.7. Detection of sugar chain in LOFG

SDS–PAGE analysis of LOFG was performed as described above. BSA (100 ng) was used as negative control, ovalbumin (100 ng) as positive control and Biotinylated SDS–PAGE Standards (Bio-Rad Inc.) were used as marker protein. The proteins in the gel were transferred to Hybond-P (GE Healthcare Co., Ltd) using semidry blotter (100 mA, 1 h) with 25 mM Tris/192 mM glycine/20% MeOH aq as blotting buffer. The membrane was washed three times with TBS-T (25 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 20 min. and then with milliQ water for three times. Glycoproteins on the membrane were then oxidized with 10 mM $NaIO_4$ in PBS (100 mM phosphate, 100 mM NaCl, pH 7.5) for 30 min at 4 °C and washed three times with PBS-T (100 mM phosphate, 100 mM NaCl, 0.1% Tween 20, pH 7.5). Blocking was performed using 2% w/v ECL Advance Blocking Agent (GE Healthcare Co.) in PBS-T and then washed three times with PBS-T. Oxidized glycoproteins were biotinylated with 0.1 mM Biotinhydrazide dissolved in 0.1 M Acetate buffer (pH 5.5) and washed twice with PBS-T. Membrane was then soaked in Streptavidin-HRP conjugate (GE Healthcare Co.) diluted with PBS-T (1/250,000) for 15 min and sufficiently washed with PBS-T. Finally chemiluminescent detection was performed using ECL advance Western Blotting Detection Kit (GE Healthcare Co.) and ECL Mini-Camera (GE Healthcare Co.) equipped with Instant Black & White Film FP-3000B Super Speedy (Fujifilm Co.).

Total protein on the membrane was detected using Colloidal Gold Total Protein Detection Kit (Bio-Rad Inc.).

3.8. Deglycosylation of LOFG

3.8.1. By *N*-glycopeptidase A. LOFG was denatured by either of the following: 2% Nonidet P-40, 2% Triton X-100, 0.1% SDS, 0.75 M NaSCN, and 6 M urea for 2 h. To the solution, *N*-glycopeptidase A (0.1 mU) was added and incubated for 24 h at 37 °C. The resulting mixture was analyzed by SDS–PAGE as described above.

3.8.2. By *N*-glycopeptidase F. LOFG was denatured by 0.5% SDS containing 0.1 M 2-mercaptoethanol for 5 min at 95 °C. To the solution, *N*-glycopeptidase F (5 U) was added and incubated for 24 h at 37 °C. The resulting mixture was analyzed by gel-filtration HPLC as described above.

3.8.3. By trifluoromethanesulfonic acid (TfOH). To the lyophilized LOFG, trifluoromethanesulfonic acid (100 μL) was added at 0 °C. After 2 h, the mixture was cooled to −20 °C and neutralized by adding 60% pyridine aq. (200 μL). The resulting solution was dialyzed with Spectra/Por Float-A-Lyzer (MWCO = 3500, Nippon Genetics Co., Ltd) and purified by Superdex 200 10/300 GL (GE Healthcare Co., Ltd). Molecular weight of the obtained protein was measured by gel-filtration HPLC [TSK-Gel SuperSW3000 (Tosoh Co. Ltd), 50 mM phosphate buffer (pH 7.0) with 0.15 M NaCl, flow rate: 0.2 mL/min, detection: 215 nm].

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